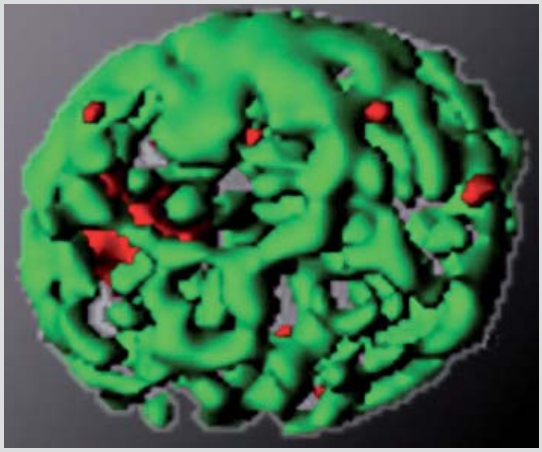

Alternate Pathways of the Early Secretory Route in Yeast

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Academic Dissertation

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Cover figure: Hsp150-R4-Bla (green, DNA red) after 10 minute exposition at 37°C in a *sec21-1* mutant.

CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following articles, and on unpublished results presented in the text. The articles are referred to in the text by Roman numerals.

I Fatal N., **Suntio T**. and Makarow M. (2002). Selective protein exit from yeast endoplasmic reticulum in absence of functional COPII coat component Sec13p. *Mol. Biol. Cell* 12:4130-40.

II **Suntio T***., Shiryaev S*. and Makarow M. ATPase activity of a yeast secretory glycoprotein causes ER exit in the absence of functional COPII component Sec24p and Sec13p. Manuscript, * Equal contribution

III **Suntio T**., Shmelev A., Lund M. and Makarow M. (1999). The sorting determinant guiding Hsp150 to the COPI-independent transport pathway in yeast. *J. Cell Sci*. 112, 3889-3998.

ABBREVIATIONS

ADP	adenosine diphosphate
ARF	ADP ribosylation factor
ATP	adenosine triphosphate
ATPase	ATP phosphatase
BFA	brefeldin A
BiP	immunoglobulin heavy chain binding protein
BLA	β-lactamase Tem 10 from *E. coli* without signal sequence
CD spectroscopy	circular dichroism spectroscopy
CDP	cytidine diphosphate
CHX	cycloheximide
COP	coat protein
CPY	carboxypeptidase Y
C-terminus	the end of a protein with a free carboxyl group
DIG	detergent-insoluble glycosphingolipid-enriched
DNA	deoxyribonucleic acid
Dol-P	dolichol phosphate
Dol-PP	dolichol pyrophosphate
EM	electron microscopy
ER	endoplasmic reticulum
ERGIC	ER Golgi intermediate compartment
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine-nucleotide exchange factor
GFP	green fluorescent protein
Glc	glucose
GlcNAc	N-acetyl glucosamine
GMP-PMP	guanylyl imidodiphosphate
COG	conserved oligomeric Golgi, a tethering complex
GPI	glycosylphosphatidylinositol
GST	glutathione S-transferase
GTP	guanosine triphosphate
GTPase	GTP phosphatase
HRP	horseradish peroxidase
Hsp	heat shock protein
kD	kilo Dalton
K_m	Michaelis-Menten constant, substrate concentration that produces a half maximal reaction rate
Man	mannose
NSF	N-ethylmaleimide-sensitive factor
N-terminal	the end of a protein with a free amino group
OST	oligosaccharyl transferase
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PKA	protein kinase A
PLD	phospholipase D
PMT	protein-mannosyl transferase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electroforesis

SNARE	soluble N-methylmaleimide-sensitive factor attachment protein receptor
SUI	subunit I
SUII	subunit II
TDP	thymidine diphosphate
TOR	target of rapamycin, a protein kinase
TRAPP	transport protein particle, a tethering complex
UDP	uridine diphosphate
UGGT	UDP-Glc glycoprotein transferase
WD40	β-propeller domain
VSV-G	vesicular stomatitis virus glycoprotein
VTC	vesicular-tubular cluster
YFP	yellow fluorescent protein

Single letter code	*Three letter code*	*Amino acid*
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Some mammalian homologs to yeast proteins

NSF	Sec18p
Rab1	Ypt1p
Rab6	Ypt6p
GBF1	Gea1p , Gea2p
BIG1, BIG2	Sec7p
ERGIC	Emp46/47p
α-COP	Ret1p
β-COP	Sec26p
γ'-COP	Sec27p
α-COP	Sec21p
ε-COP	Sec28p
δ-COP	Ret2p
ζ-COP	Ret3p

"*wild type cells*" used in this book, mean cells without known secretion mutations.

SUMMARY

The diversity of functions of eukaryotic cells is preserved by enclosing different enzymatic activities into membrane-bound organelles. Separation of exocytic proteins from those which remain in the endoplasmic reticulum (ER) casts the foundation for correct compartmentalization. The secretory pathway, starting from the ER membrane, operates by the aid of cytosolic coat proteins (COPs). In anterograde transport, polymerization of the COPII coat on the ER membrane is essential for the ER exit of proteins. Polymerization of the COPI coatomer on the *cis*-Golgi membrane functions for the retrieval of proteins from the Golgi for repeated use in the ER.

The COPII coat is formed by essential proteins; Sec13/31p and Sec23/24p have been thought to be indispensable for the ER exit of all exocytic proteins. However, we found that functional Sec13p was not required for the ER exit of yeast endogenous glycoprotein Hsp150 in the yeast *Saccharomyces cerevisiae*. Hsp150 turned out to be an ATP phosphatase. ATP hydrolysis by a Walker motif located in the C-terminal domain of Hsp150 was an active mediator for the Sec13p and Sec24p independent ER exit. Our results suggest that in yeast cells a fast track transport route operates in parallel with the previously described cisternal maturation route of the Golgi. The fast track is used by Hsp150 with the aid of its C-terminal ATPase activity at the ER-exit. Hsp150 is matured with a half time of less than one minute. The cisternal maturation track is several-fold slower and used by other exocytic proteins studied so far.

Operative COPI coat is needed for ER exit by a subset of proteins but not by Hsp150. We located a second active determinant to the Hsp150 polypeptide's N-terminal portion that guided also heterologous fusion proteins out of the ER in COPII coated vesicles under non-functional COPI conditions for several hours. Our data indicate that ER exit is a selective, receptor-mediated event, not a bulk flow. Furthermore, it suggests the existence of another retrieval pathway for essential reusable components, besides the COPI-operated retrotransport route. Additional experiments suggest that activation of the COPI primer, ADP ribosylation factor (ARF), is essential also for Hsp150 transport. Moreover, it seemed that a subset of proteins directly needed activated ARF in the anterograde transport to complete the ER exit.

Our results indicate that coat structures and transport routes are more variable than it has been imagined.

1 INTRODUCTION

Humans have used yeast (*Saccharomyces cerevisiae*) in their households for several thousands of years. The earliest findings are more than 7000 years old from a possible wine container (McGovern *et al.* 1996). The concept of micro-organisms being responsible for fermentation was developed in the 19th century by Louis Pasteur. Yeast genetics started in the 1930s from the studies of Winge, and later of Lindegren, which led to the isolation of the *S. cerevisiae* strain S288C, now used in laboratory studies (Mortimer and Johnston 1986). Its genome was the first eukaryotic one to be completely sequenced (reviewed by Goffeau *et al.* 1996).

Baker's yeast is commonly used as a eukaryotic cell model. It is a small unicellular organism which can be grown as a haploid or a diploid strain on defined, simple and cheap media. It is generally regarded as a safe organism, the use of which does not pose ethical concerns. Genetic manipulation of yeast is easy and fast, and has opened opportunities to study the function of gene products of other eukaryotes in the yeast system. Many basic cellular functions are conserved from yeast to man and homologous genes can often complement each other.

Using animal cells as a model Jamieson and Palade (1967) postulated a theory of newly synthesized proteins proceeding through intracellular membrane-bound compartments, leading to protein accumulation into secretion granules in pancreatic acinar cells. Yeast genetics has been extremely valuable also in studies of the secretory pathway. Isolation of 23 different complementation groups of mutants that conditionally block the intracellular protein transport to various compartments (Novick *et al.* 1980) activated secretion pathway studies in yeast. According to current understanding, the secretory pathway is needed by proteins that are finally localized to the exterior of the cell, the plasma membrane, vacuole or endosomes (Fig. 1). Proteins resident in the Golgi or the endoplasmic reticulum (ER) begins the same pathway. The secretory pathway starts as the polypeptide enters the ER lumen, or is integrated into the ER membrane. In the ER it experiences chaperone-assisted folding, disulfide bonding and glycosylation. Correctly folded proteins are sorted into protein-coated lipid vesicles that pinch off from the ER membrane and fuse with the *cis*-Golgi membrane. In other subcompartments of the Golgi, further modifications of the protein-linked carbohydrate side chains as well as proteolytic cleavages may take place. According to the current view, retrograde transport from the later to the earlier Golgi subcompartment or to the ER stabilizes the membrane flow, modifies the enzyme content of the compartments, and returns escaped or reusable proteins back to the ER. From the Golgi the exocytic proteins are sorted to different destinations in the cell. The time from protein synthesis to the delivery of the protein to the exterior of the cell takes about ten minutes in yeast. The generation time of *S. cerevisiae* under optimal conditions is about 90 minutes. Based on subcellular localization studies of the yeast proteome approximately 13% of the yeast proteins have been estimated to be part of the secretory pathway (Kumar *et al.* 2002).

1.1 Endoplasmic reticulum

The yeast ER like that of all eukaryotes is a continuous membrane structure limited by a phospholipid bilayer shaping a joint lumenal space. In higher eukaryotes, using

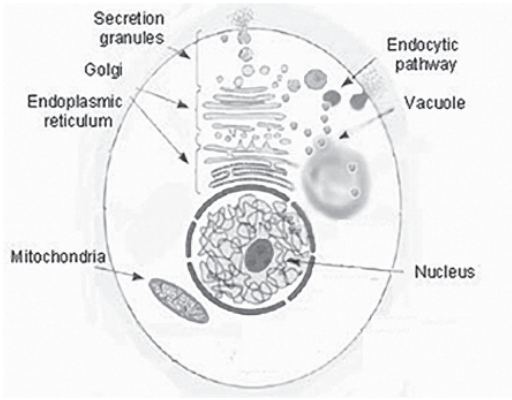

Figure 1. A schematic picture of the principal secretion pathway routes of the eukaryotic cell.

fluorescence recovery after irreversible photo bleaching (FRAP) and green fluorescent protein (GFP) tagged proteins, the ER has been found to be one single continuous organelle. It was demonstrated that proteins are able to rapidly diffuse throughout the ER (Cole *et al.* 1996). The ER is a continuum with the outer nuclear membrane. It extends to the periphery of the cell forming sheets and intersecting dynamic tubular structures connected to the actin cytoskeleton (Prinz *et al.* 2000). Formation of tubular structures and their stabilization is dependent on two membrane deforming hairpin-structure containing proteins that give shape to the membrane (Voeltz *et al.* 2006). The ER has many activities including biosynthesis of lipids for constructing new membranes, folding of new proteins including their oligomerization, quality control and targeting of misfolded proteins for degradation. The ER is also a site for synthesis of protein and lipid-linked carbohydrates, as well as of the glycosylphosphatidylinositol (GPI)-anchor and its ligation to target proteins. Morphologically the ER can be divided into rough and smooth ER, depending on the presence of ribosomes on the cytosolic face.

1.1.1 Protein folding inside the ER

Proteins enter into the ER either co- or post-translationally directed usually by N-terminal, short, quite hydrophobic amino acid sequence, called a signal peptide. Newly synthesized polypeptides pass across the ER membrane through a protein pore, the translocon, where Sec61p or its homolog forms the channel (see Wilkinson *et al.* 1997). As soon as the peptide emerges from the translocon channel, the signal peptide is cleaved off and the protein starts to fold, domain by domain, to its correct three-dimensional structure.

Folding of proteins *in vitro* to functional, energetically favourable states is possible, as Anfinsen and coworkers (1961) showed by studying the re-folding of reduced bovine pancreatic ribonuclease, following its spectral properties for several hours. They concluded that “the information for the correct pairing of half-cystine residues in disulfide linkage, and for the assumption of the native secondary and tertiary structures, is contained in the amino acid sequence itself.” In the ER proteins fold according to the same physical laws as in the test tube, dictated by the amino acid primary sequence. Generally, folding of secondary structures occurs mainly by formation of hydrogen bonds. α-Helices and β-sheets are formed locally in a linear sequence. Thereafter these structures fold and twist to form a tertiary structure.

Cells have evolved a refined and essential machinery of proteins, called folding enzymes and chaperones, which assist the folding of newly made polypeptides. The importance of proper protein folding is highlighted by the fact that a number of human diseases result at least partially from protein misfolding events in the ER, such as prion diseases (Aguzzi *et al.* 2004), cystic fibrosis (Sharma *et al.* 2004), trypsin deficiency (Lawless *et al.* 2004) and a familial hypercholesterolemia (Li *et al.* 2004). In many diseases (e.g. cardiovascular diseases, diabetes, cancer, viral infections) defective protein folding induces ER stress either as a cause or a secondary symptom that can lead to apoptosis (see Zhao and Ackerman 2006). The ER is a highly specialized folding compartment containing millimolar concentrations of Ca^{2+}, the co-factor of many chaperones. The ER has a relatively high content of oxidized glutathione (GSSG:GSH 1:3) as compared with the cytosol (1:100), which allows disulfide bridge formation (see Frand *et al.* 2000). If authentic disulfide bond formation is prevented for example with reducing agents, secretion of some proteins is blocked at the level of ER exit (Braakman *et al.* 1992, Jämsä *et al.* 1994).

1.1.1.1 Folding enzymes

Yeast protein disulfide isomerase (Pdi1p) is an essential, abundant ER resident protein, composed of four thioredoxin motifs (CXXC) located near the N- and C-termini, facilitating thiol-disulfide exchange (reviewed by Tu and Weissman 2004; Chakravarthi *et al.* 2006.). In addition, Pdi1p contains two internal, non-active cysteines forming a stable disulfide that destabilizes the N-terminal active site disulfide, making Pdi1p

a better oxidant (Wilkinson *et al.* 2005). Pdi1p catalyzes the oxidation, reduction and isomerization of incorrectly formed disulfide bonds by forming a mixed disulfide between the Pdi1p and the folding intermediate, until the correct bond is formed. Correct bonds allow the protein to proceed folding properly (Wilkinson and Gilbert 2004; Fig. 2). Pdi1p is re-oxidized by the conserved ER membrane protein oxidoreductase (Ero1p, Frand and Kaiser 1998, Pagani *et al.* 2001). Ero1p in turn is oxidized by molecular oxygen (Tu and Weissman 2002). Also other redundant Pdi1p homologs exist in yeast, namely Mpd1p, Mpd2p Eug1p and Eps1p. Overexpression of Mpd1p can fully replace Pdi1p functions, but the other homologs except Eps1p have low oxidative refolding activities. (Norgaard *et al*. 2001).

Another folding enzyme, cyclophilin, catalyzes time consuming *cis-trans* conversions of peptide-proline bonds to the *trans*-form. The yeast ER resident cyclophilin, Fkb2p, is a membrane-bound enzyme, the expression of which is induced by protein accumulation in the ER lumen (Nielsen *et al.* 1992). The importance of this protein family to mammals is described in a study where the cyclophilin family member FKB12.6 seemed to be responsible for keeping calcium pumping ryanodine receptors stabilized in muscle cells. A mouse lacking FBK12.6 died of cardiac attack during exercise, due to destabilization of the calcium pump (Wehrens *et al*. 2004).

1.1.1.2 Chaperones

The term molecular chaperone was applied to proteins, which prevented wrong interactions between histones and DNA, by Laskey and coworkers (1978). The concept of a chaperone was invented after studies of mitochondrial Hsp60 (Cheng *et al.* 1989) and correct folding supervisors of rubisco subunits (Ellis 1987). The chaperone term is used for proteins that assist the folding of target proteins without an impact on the final pattern of the fold. Chaperones usually belong to heat-induced proteins that are subdivided into Hsp60, Hsp70, Hsp90, Hsp100, and small Hsp subclasses.

An ER-located yeast chaperone, Kar2p, was first identified as a *kar2* mutant defective in fusing nuclei during mating (Polaina

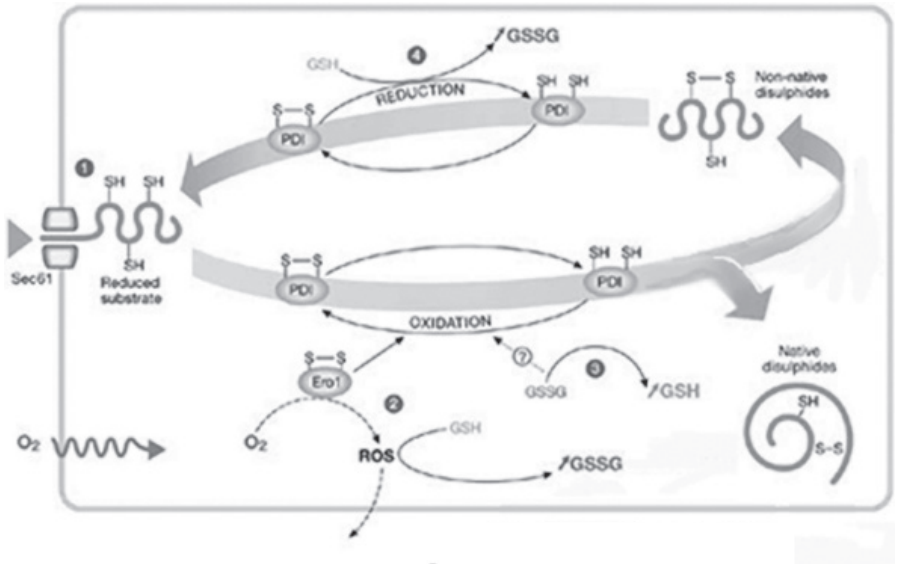

Figure 2. A schematic picture of disulfide isomerase (PDI) cycle in the ER. After translocation into the ER, oxidation of reduced sulfhydryl groups of folding intermediate polypeptides are catalysed by help of PDI internal sulfhydryl bridges. The same enzyme also catalyses reshuffling of bonds by reduction and forming covalently bonded folding intermediates until the correct fold is found. Modified from Chakravarthi *et al.* 2006. Reprinted by permission from Macmillan Publishers Ltd: Nature reviews Molecular Cell Biology. Copyright 2006, Nature publishing Group.

and Conde 1982). Kar2p expression is constitutive, but induced by heat and ER stress (Gething 1999). Deletion of *KAR2* gene is lethal, but heterologous expression of the mammalian counterpart, BiP, can support growth (Normington *et al.* 1989). BiP was isolated as an immunoglobulin heavy chain binding protein (Haas and Wabl 1983). BiP is temporarily bound to partially unfolded, unassembled polypeptides (Gething and Sambook 1992), and released from folding intermediates by ATP-ADP cycling. The preferential binding site in the folding intermediate is an extended stretch of seven amino acids where every second residue is either hydrophobic or bulky (Flynn *et al.* 1991, Blond-Elguindi *et al.*1993). Kar2p/BiP belongs to the Hsp70 family. It has a conserved ATP-binding domain in the N-terminal region, a central peptide-binding domain, and a C-terminal lid domain that locks peptide entry and release (Palleros *et al.* 1993, Zhu *et al*. 1996). Many ATP- and GTP-binding proteins bind the nucleotide through a P-loop fold having a conserved Walker A-motif (GXXXXGKT/S) where the lysine residue (K) binds directly to the γ-phosphate group (Walker *et al.* 1982). ATP-binding changes the conformation, and peptide binding stimulates the ATPase activity in Kar2p/BiP (Flynn *et al.* 1989). In ATP-bound form the chaperone exhibits a fast exchange rate for peptides. ADP-ATP cycling leads to binding and releasing of the peptide, which finally finds its correct fold. A failure to fold results in permanent association with BiP and degradation, since a hydrophobic sequence on the surface of the misfolded protein leads to aggregation (Ellgaard and Helenius 2003).

The steady-state turnover rate of the Hsp70 ATPase is slow (between 0.02 and 0.2 min^{-1}). The presence of an unfolded hydrophobic peptide stimulates the ATPase activity 2–10-fold (Flynn *et al.* 1989, Jordan *et al.* 1995). *In vivo* the ATPase activity is up-regulated by cofactors that contain a domain homologous to bacterial DnaJ-family members. Scj1p is one of several DnaJ homologues in the yeast ER, and a possible cofactor for Kar2p. Its deletion leads to hypersensitivity towards the antibiotic tunicamycin, and transport defects in N-glycosylation mutants (Silberstein *et al.* 1998).

ER stress is a condition where unfolded proteins accumulate in the ER lumen due to denaturation of proteins by heat, chemical stress or mutation. Prolonged stress may lead to apoptosis (see; Zhang and Kaufman 2006, Zhao and Ackerman 2006). Stress awareness is regulated through Kar2p, which binds to the lumenal parts of the Ire1p kinase, keeping it as a monomer. If the protein load in the ER lumen is such that no Kar2p is available to bind Ire1p, Ire1p oligomerizes and becomes auto-phosphorylated. This leads to activation of the transcription factor Hac1p regulating ER chaperone production (Shamu and Walter 1996, Cox and Walter 1996).

Kar2p binding to normal folding intermediates is transient and difficult to study since defects in Kar2p function affect already the translocation of proteins into the ER (Brodsky *et al.* 1995). Holkeri and coworkers (1998) studied the folding of heterologous reporter proteins in a temperature sensitive Kar2p strain by preventing disulfide bond formation with a reducing agent *in vivo*. Thereafter Kar2p was inactivated by shift of the cells to the sensitive temperature by heat treatment. A fusion protein containing the nerve growth factor receptor-ectodomain ($NGFR_e$) that harbours 24 cysteines refolded without Kar2p activity after washing out the reducing agent. In contrast, a β-lactamase fusion protein with a single disulfide bond needed Kar2p for folding. Thus, the requirement of Kar2p for folding seemed to be substrate-specific and is probably dictated by the presence of an N-glycan in the N-terminal part of the polypeptide. If there is a glycan, the polypeptide is folded most probably by other folding enzymes than Kar2p, such as the calnexin cycle enzymes (Molinari and Helenius 2000).

A nonessential Cne1p chaperone was revealed as it enhanced the refolding of a heat denatured protein *in vitro* in a concentration-dependent manner. In addition, the chaperone function of Cne1p was greatly affected in the presence of monoglucosylated oligosaccharides in the substrate protein which specifically bound

to the Cne1p lectin site (Xu *et al.* 2004). Cne1p is a yeast homolog of mammalian calnexin, which binds trimmed N-glycans of folding intermediates, preventing protein aggregation. A calnexin-bound form of a folding intermediate serves as a substrate for other folding enzymes, like the PDI homolog ERp57. Further trimming of the N-glycan releases the folding substrate from calnexin, followed by ER exit of the correctly folded protein. An unfolded polypeptide is still recognized by a UPD-glucose glycoprotein transferase enzyme (UGGT) making it a substrate for the calnexin/ calreticulin cycle again (Fig. 3; Parlati *et al.* 1995, Helenius and Aebi 2004). The calnexin cycle is also linked to a degradation pathway of permanently misfolded proteins. After unsuccessful folding cycles unfolded proteins are marked by a slow enzyme harbouring α-1,2-mannosidase activity, and donated to the calnexin-connected eight mannose binding EDEM (Htm1) protein to be targeted for degradation (Oda *et al.* 2003, Jakob *et al*. 2001).

Glucose trimming enzymes and the glucose transferase homolog are identified also from yeast suggesting the existence of a similar glycoprotein folding and sensing cycle in the yeast ER (Trombetta *et al.* 1996). Yeast Mpd1p (Pdi1p homolog) is connected to Cne1p, which increased Mpd1p reductivity (Kimura *et al*. 2005) and thereby sulfhydryl isomerization activity.

Membrane protein-specific chaperones have been described. They facilitate the correct folding of their substrate without an influence on other proteins. Shr3p supports the folding of amino acid permeases by preventing aggregation of transmembrane domains during the folding process. Similarly, also other multi-transmembrane

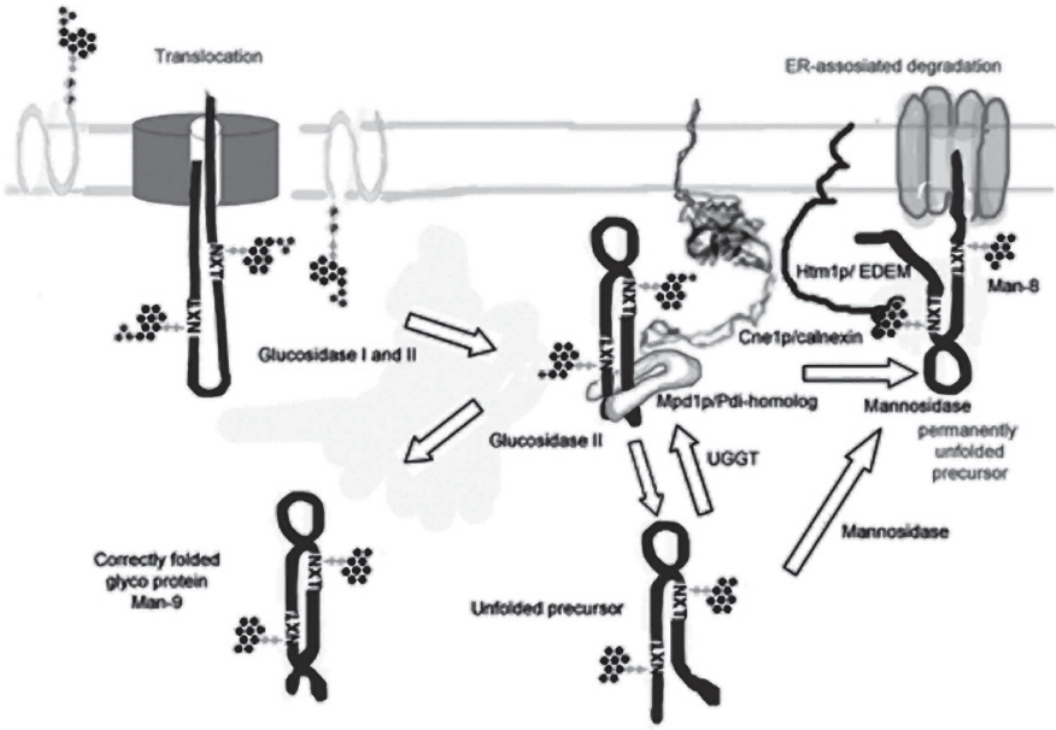

Figure.3. Schematic picture of protein folding in the calnexin cycle. After polypeptide translocation and glycosylation, glucosidase I and II trim N-glycan to mono glucosylated form that is binding substrate to Cne1p/ calnexin chaperon. While bound to calnexin sulfhydryl bridges are catalyzed by PDI/Mpd1p and last glucose unit is removed. Correctly folded protein continues its secretion. Unfolded proteins are recognized by UGGT and re-glucosylated to a substrate of calnexin, again. Slow enzyme activity also removes a mannose unit and long time in the ER/ calnexin bound folding intermediate is guided to degradation.

proteins have their own folding supporters (like hexose transporters, phosphate transporters, and chitin synthase-III; Kota and Ljungdahl 2005).

1.1.2 Protein glycosylation in the ER

Most secretory proteins are glycosylated. Glycans are involved in folding, stabilization and sorting of proteins as well as in signalling events (see Strahl-Bolsinger *et al.* 1999, Helenius and Aebi 2001, Hebert *et al.* 2005). Glycans are covalently bound to an amino (N-glycosylation) or a hydroxyl group (O-glycosylation) of specific amino acid residues in the ER lumen. N-glycosylation in eukaryotic cells follows a highly conserved pathway (see Burda and Aebi 1999). Also yeast-like O-mannosylation in mammalian ER is found (Jurado *et al.* 1999). Yeast has been a model system for studies of ER-associated glycosylation, and for studies of the molecular basis of human disorders (Aebi and Hennet 2001). Some inherited recessive human diseases, such as muscular dystrophy, congenital disorders, and mental retardation have been associated with defective O-glycosylation of α-dystroglycan, a membrane protein that connects the actin cytoskeleton to the extracellular matrix (van Reeuwijk *et al*. 2005). In Finland, we have in our gene pool one rare example of an O-glycosylation-related genetic disease, MEB (muscle-eye-brain disease) that is mapped to POMGnT1-mutations preventing processing of single mannose-containing glycans in the Golgi (see Endo and Toda 2003). Mutations in dolichyl phosphorylase lead to profound muscular hypotonia, inflammation and cardiac failure around the age of six months (Kranz *et al.* 2007).

Both the N- and O-glycosylating glycans are initiated at the cytoplasmic face of the ER membrane, when a hexose is delivered from nucleotide-activated monosaccharide donors, UDPGlcNac, GDP-Man or UDP-Glc, to dolichol phosphate carrier (Dol-P) (see Burda and Aebi, 1999). Yeast Dol-P is a lipid molecule containing around 16 polyisoprenols (see Schenk *et al*. 2001), making the chain length about three times longer than the membrane bilayer thickness.

A cytoplasmic core glycan is assembled from two N-acetylglucosamine (GlcNAc) and five mannose (Man) residues bound to the dolichol pyrophosphate (Dol-PP)-carrier (see Burda and Aebi, 1999). The first N-acetylglucosamine transfer reaction can be inhibited with an antibiotic, tunicamycin, leading to a total block of N-glycosylation (Elbein *et al.* 1987). Cytoplasmic Man5GlcNAc2-PP-Dol is then translocated across the ER membrane by the action of the flippase Rft1p, whereafter the oligosaccharide faces the lumen of the ER (Helenius *et al.* 2002). In the ER, the biosynthesis continues to completion by the serial actions of Alg (asparagine linked glycosylation) proteins using Dol-P-Man or Dol-P-Glc as a donor, resulting in Glc3Man9GlcNAc2-PP-Dol (Glc; glucose, see Burda & Aebi, 1999).

The pre-assembled core oligosaccharide is then transferred from dolichol pyrophosphate to a newly synthesized polypeptide by the ER-resident oligosaccharyl transferase complex (OST, Knauer and Lehle 1999). OST is located near the translocon (Wang and Dobberstein 1999, Nilsson *et al.* 2003) and transfers the core oligosaccharide to the amino group of the asparagine residue of the consensus sequence N-X-S/T, where X may be any amino acid except proline (see Kukuruzinska *et al.* 1987; Fig. 4).

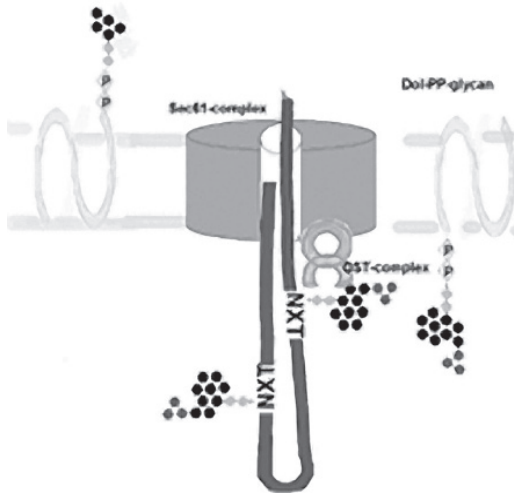

Figure 4. Glc3Man9GlcNAc2-PP-Dol is the donor of the oligosaccharide in N-glycosylation.

Soon after the oligosaccharide precursor is transferred to the folding protein intermediate, it is trimmed, whereafter the glycosylated polypeptide probably enters the calnexin folding cycle. The effect of glycosylation deficiency is protein-dependent; some proteins aggregate and are targeted for degradation, whereas others are not influenced and remain secretion competent (Helenius and Aebi 2001).

Yeast O-linked glycans are linear chains of normally from one to five mannose residues. GDP-mannose is first loaded on Dol-P by Dpm1p, on the cytoplasmic face of the ER membrane. In a temperature-sensitive *dpm1* mutant both O-mannosylation and the elongation of N-glycans is lost, indicating a total dependence on functional Dpm1p (Orlean 1990). How Dol-P-Man is flipped to the luminal side is unknown at the moment. Transfer of the first mannose residue to a serine or a threonine residue of substrate proteins is catalyzed by a family of ER-located protein mannosyltransferases encoded by seven genes (*PMT1-7*, Strahl-Bolsinger *et al.* 1999).

The enzymes are divided into Pmt1, Pmt2 or Pmt4 sub-families, of which Pmt1 and Pmt2 family members form functional heterodimeric complexes. Pmt4p, a single member of the subfamily, forms homodimers (Girrbach and Strahl 2003). Triple deletions of *PMT1, 2, 4* or *PMT2, 3, 4* genes are lethal (Gentzsch and Tanner 1996). Analyzing *in vivo* mannosylation of yeast proteins in the *pmt* mutants pointed out that those different O-mannosyl transferases had different protein substrates. Six out of ten substrate proteins tested (chitinase, a-agglutinin, Kre9p, Bar1p, Pir2p/Hsp150, Kre1p, Kre9p) were hypo-glycosylated in *pmt1* and *pmt2* mutants, while four were unaffected (Kex2p, Axl2p, Gas1p, Fus1p). Pmt4 was responsible for glycosylation initiation in the ER lumen for the rest of the proteins (Gentzsch and Tanner 1997, Sanders *et al.* 1999, Proszynski *et al.* 2004). The reason behind the substrate selection is not known. Threonine seemed to be a better acceptor *in vitro* than serine. Proline in the peptide chain upstream of the acceptor group favoured glycosylation (Strahl-Bolsinger *et al.* 1999). O-Mannosylated proteins usually contain several potential sites in clusters. The peptide might then adopt a stiff and extended conformation (Jentoft 1990). While Pmt family transferases initiate O-glycosylation in the ER lumen, elongation continues in Golgi due to different enzymes (see Strahl-Bolsinger *et al.* 1999; Fig. 5).

1.1.3 Protein sorting in the ER

Fully folded and assembled exocytic proteins are separated from the ER-resident ones for the anterograde transport into protein-coated vesicles designated COPII vesicles (see Tang *et al.* 2005, Mancias and Goldberg 2005), or into tubular elements (see Watson and Stephens 2005). The bulk flow model suggests that cargo proteins are packaged into transport vesicles without concentration. The secretory cargo would be concentrated after the ER exit due to the return of ER-resident proteins (Wieland *et al.* 1987). This appears to be true for example in some mammalian exocrine cells that are specialized for secretion (Martinez-Menarguez *et al.* 1999).

An opposite model where secretory proteins harbouring specific sorting signals are enriched in transport vesicles before leaving the ER, got support from immuno electron microscopic studies, where a viral membrane protein was ten times more concentrated at ER exit sites as compared to other parts of the ER membrane (Balch *et al.* 1994). In receptor-mediated export, proteins could be selected to COPII-coated vesicles by direct interaction with a cargo-recognizing transmembrane protein (Kuehn *et al.* 1998). Cumulative evidence supports the existence of specific cargo receptors that cycle between the ER and the Golgi (see Barlowe 2003). Furthermore, a soluble

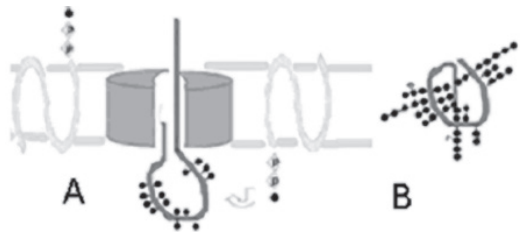

Figure 5. Initiation of O-mannosylation in the ER lumen (A). An example of yeast specific Golgi modifications to O-glycosylated proteins (B).

cargo protein, pro-α-factor, was enriched approximately 20-fold in ER-derived vesicles, relative to bulk flow markers in the yeast ER (Malkus *et al.* 2002).

Several unassembled secretory protein complex units are retained in the ER by chaperones until assembly is completed. Mechanisms for retention of ER-resident proteins within the ER are not well known. ER localization of human Ero proteins seemed to be dependent on saturable covalent sulphide interactions with ERp44, an isomerase homolog (Otsu *et al.* 2006). Large, ER-localized multiprotein complexes, including most of the molecular chaperones (except those of the calnexin cycle), are found in several mammalian cell lines. The chaperone complex is associated with unassembled immunoglobulin heavy chains in immune system cells. However, the complex was present also in the absence of new protein synthesis, indicating that complex formation is a possible retention mechanism for ER-resident soluble chaperones (Meunier *et al.* 2002).

For escaped ER resident proteins specific receptor-mediated retrieval mechanisms have evolved. These include retrieval from the Golgi to the ER of soluble KDEL/ HDEL- sequence containing proteins, as well as certain transmembrane proteins (Pelham and Munro, 1993, Sato et al. 2003).

One option for the sorting mechanisms is exclusion from budding vesicles. The exclusion mechanisms for membrane proteins may include a variation in lipid chain length leading to a local thickness difference. The thickness of the membrane increases progressively towards the cell surface, due to enrichment of cholesterol and sphingolipids and this might be a sorting mechanism for membrane proteins (see Munro, 1998). Or, the lipid composition may vary locally, like in detergent-insoluble glycosphingolipid-enriched (DIG) liquid order formation. In yeast, ergosterol synthesis as well DIG formation starts in the ER membranes (see Parks *et al*. 1995). DIGs play a part in GPI-linked protein sorting at ER exit and the ER-resident protein Sec61p is excluded from DIG structures (Bagnat *et al.* 2000). Proteins that regulate the exclusion process are unknown. Some genes (*BST1, BST2/EMP24, and BST3*) have been identified, the deletion of which led to enhanced secretion of ER-resident chaperones Kar2p and Pdi1p (Elrod-Erickson and Kaiser 1996). Also in an *ERD1* deletion strain Kar2p was secreted to the medium like itsHDEL-deletion variant (Hardwick *et al*. 1990). Erd1p is an ER-located transmembrane protein with unknown function. Other retention-specific mutants have been isolated such as *cis* prenyltransferase, the key enzyme in dolichol synthesis. Possibly, protein retention in the ER was indirectly affected by the defect in glycosylation. Or, dolichol has unexpected roles in protein retention (Sato *et al*. 1997).

Several or all of these mechanisms may be coordinated to maintain the identity of organelles.

1.1.3.1 ER exit sites

The exocytic cargo departs from the ER exit sites. They are characterized by COPII coat component covered vesicle budding areas, and lack ribosomes, which are widespread on the surface of the rough ER (Bannykh *et al.* 1996). Studies in mammalian cells harbouring a thermo-reversible folding mutant of a viral glycoprotein (tsO45VSV), showed ER exit sites to be a defined but still functionally connected part of the ER (Mezzacasa and Helenius 2002). Normally, at 10 ºC transport in mammalian cells is blocked and newly synthesized proteins accumulate in the ER. Under such conditions tsO45VSV resided at the ER exit sites together with COPII components where ER chaperones were lacking. Raising the temperature from 10 ºC to almost 40 ºC resulted in misfolding of the tsO45VSV protein, and retrieved chaperone association. On the other hand, transport of correctly folded tsO45VSV to the next compartment (vesicular tubular clusters: VTC/ intermediate compartment: IC/ ERGIC) by raising the temperature from 10 ºC to 15 ºC prevented chaperone association (Mezzacasa and Helenius 2002).

ER exit sites seemed to be morphologically different in baker's yeast and *Pichia pastoris,* as visualized with GFP-

fusions to Sec13p, a component of the COPII coat, and with another membrane protein, Sec12p-myc, colocalized to exit sites. *P. pastoris* had a few concentrated cargo exit sites, while in baker's yeast dispersed exit sites spanned the entire ER (Rossanese *et al.* 1999). Differences between ER exit sites are also believed to influence the localization of the Golgi. Sec12p localization at ER exit sites in *P. pastoris* seemed to depend on the interactions of its cytoplasmic domain, because its deletion led to a diffuse Sec12p staining, while ER exit sites were not influenced (Soderholm *et al.* 2004). A substitutional mutation in the peripheral membrane protein Sec16p resulted in temperature-dependent diffusion of *P. pastoris* ER exit sites and dispersed Golgi structures (Connerly *et al.* 2005).

1.2 Protein transport

Exocytic soluble proteins and the lumenal parts of membrane proteins are separated from the cytosol by a lipid layer as soon as they translocate across the ER membrane. Transport of the cargo from the ER to other membrane-enclosed compartments normally requires a lipid vesicle pinching off from the donor membrane. Fusion of the vesicle with the acceptor membrane delivers the cargo to the next organelle (Fig. 6). The pinching step is assisted by cytosolic proteins that polymerize onto the membrane, and help capture the cargo molecules. Cytosolic proteins that polymerize and form coats on membranes include clathrin, COPI and COPII proteins (see Bonifacino and Glick 2004). Many different protein factors are needed to ensure the correct timing and placing of tethering and fusion, and to prepare the factors for a new cycle of fusion.

1.2.1 Basic components in COPII coat formation

The basics for understanding vesicular transport in yeast came from the analysis of 23 different conditional secretion mutant groups. Following transport of marker proteins, a set of mutants were discovered that blocked transport between the ER and the Golgi (Novick *et al.* 1981). Morphological analysis distinguished mutants where vesicle formation from the ER was blocked resulting in ER enlargement (*sec12, sec13,*

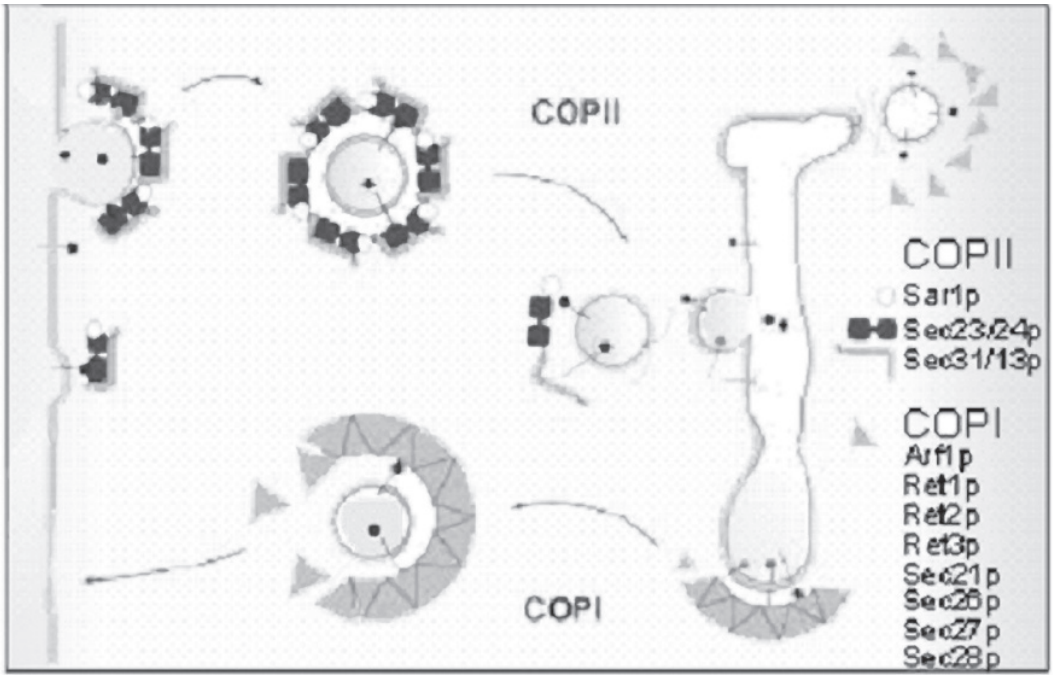

Figure 6. Vesicle forming coats, COPI and COPII, known to operate in the early secretion pathway.

sec16, *sec23*), from mutants accumulating 50 nm vesicles (*sec17, sec18, sec22)* (Kaiser and Schekman 1990). Many of these genes were isolated as single copy suppressors of temperature-sensitive growth (*SEC12*; Nakano *et al.* 1988, *SEC23*; Hicke and Schekman 1989, *SEC13*; Pryer *et al.* 1993, *SEC16*; Espenshade *et al.* 1997).

Other genes were found to be multicopy suppressors of the isolated mutants, like *SAR1*, which suppresses the growth defect of the *sec12-4* mutant (Nakano *et al.* 1989). Sar1p turned out to be an essential GTP-binding protein of 21 kD with homology to the Ras-proteins' GTP-binding domain (Fig 7A). Sar1p functions as a regulator of vesicular traffic from the ER. Since Ras-family proteins cycle between an activated GTP and membrane-bound form, and a cytoplasmic inactive GDP-bound form (see Segev 2001), the GDP exchange and GTPase accelerator factors (GEFs and GAPs) were examined for Sar1p. Sec12p turned out to be a GEF for Sar1p. Sec12p is a glycosylated type II transmembrane protein of the ER, and harbours a GDP dissociation activity towards Sar1p-GDP in its cytoplasmic N-terminal domain (Barlowe and Schekman 1993). The cytoplasmic domain is predicted to fold as a β-propeller WD40 structure, a common structure functioning in protein-protein interactions (Chardin *et al.* 2002, Fig. 7B). The GTPase activity trigger is located in Sec23p, which is one of the COPII coat proteins (Fig. 8). The presence of isolated Sec23p could specifically stimulate the Sar1p GTPase activity about tenfold *in vitro* (Yoshihisa *et al.* 1993).

Sec16p was shown to be an essential hydrophilic tightly bound peripheral membrane-associated protein of 240 kD (Espenshade *et al.* 1997). Genetic interaction studies (double mutant lethality) clearly placed Sec16p to the COPII vesicle formation, since *sec16-1* was not able to form colonies in combination with *sec12-1, sec13-1* or *sec23-1* mutations (Kaiser and Schekman 1990). Moreover, Sec16p was found in isolated vesicles (Espenshade *et al.* 1997).

1.2.2 *In vitro* studies of COPII vesicle formation

More information on the functions and essentiality of the COPII proteins came from *in vitro* studies, where a radioactively labelled reporter, the precursor of α mating factor (α factor) was posttranslationally inserted into the ER membrane harvested from different mutant strains and mixed with cytosols (for example Ruohola *et al.* 1988, Rexach and Shekman 1991, Hicke *et al.* 1992, Barlowe *et al.* 1993, Pryer *et al.* 1993, Salama *et al.* 1993). From these experiments the basic requirements where clarified. The presence of GTP-nucleotide, Sec23/24p- and Sec13/31p-complexes

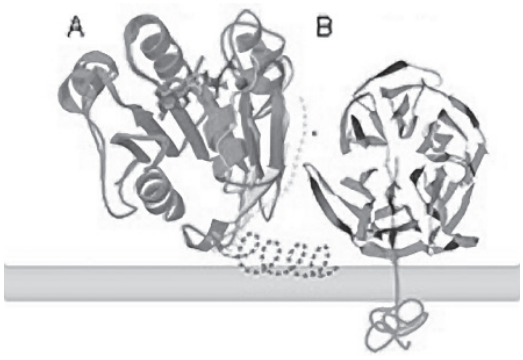

Figure 7. Structure of the small GTPase Sar1p (A) and its exchange factor Sec12p (B). Modified from Gurkan *et al.* 2006. Reprinted by permission from Macmillan Publishers Ltd: Nature reviews Molecular Cell Biology Gurkan *et al*. 2006, Copyright 2006, Nature publishing Group

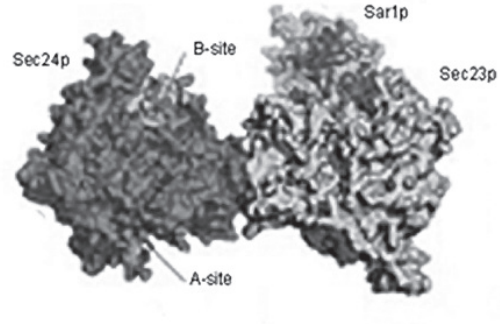

Figure 8. The bow-tie like structure of the COPII coat components Sec23/24p with Sar1p. Different known cargo binding sites are marked. Modified from Gurkan *et al.* 2006. Reprinted by permission from Macmillan Publishers Ltd: Nature reviews Molecular Cell Biology Gurkan *et al*. 2006, Copyright 2006, Nature publishing Group

and Sar1p were essential for the budding reaction to occur.

Identification of Sec24p first as a 105 kD protein that co-isolated in a gel filtration purification protocol in a 400 kD complex with Sec23p, led to *in vitro* experiments, where its essentiality for the ER-derived vesicle formation was discovered (Hicke *et al.* 1992). *SEC24* was cloned as a suppressor of the temperature sensitive *sec24-1* mutation (Gimmeno *et al.* 1996). Likewise, another protein (p150) was isolated in a 700 kD complex with Sec13p. The respective gene was later cloned by PCR with primers based on the peptide fragments of p150, and named *SEC31* (Pryer *et al.* 1993, Salama *et al.* 1997).

Barlowe and coworkers (1994) were also able to reproduce a budding reaction with purified protein components (Sar1p, Sec13/31p- and Sec23/24p-complex) without added cytosol. Using a nonhydrolyzable GTP-analogue, guanylyl imidodiphosphate (GMP-PMP), a 10 nm thick protein coat was visible in EM on formed vesicles. They designated these vesicles COPII-coated vesicles. Inhibition of fusion of the ER-derived vesicle with a target membrane led to the isolation and characterization of cargo proteins from *in vitro*-produced vesicles including the α factor, SNAREs (Soluble NSF attachment protein receptors) Bet1p, Bos1p, Sec22p and twelve abundant membrane associated proteins named ERV-proteins (ER-derived vesicle, Rexach *et al.* 1994).

The sequential binding of coat components was studied using synthetic liposomes containing negatively charged phospholipids. Sar1p-GTP bound to liposomes, and first recruited the Sec23/24p-compex whereafter the Sec13/31p-complex was bound to the previous components. If GMP-PMP was used, it led to vesicle budding (Matsuoka *et al.* 1998). The morphological arrangements of the coat structure were also studied in the *in vitro* liposome system (Matsuoka *et al.* 2001). Cross-linking of Sar1p to phospholipid probe on the liposomes was enhanced by the presence of GMP-PMP and cross-linking of the Sec23/24p complex was totally dependent on GMP-PMP (Matsuoka *et al.* 2001). Sec13/31p in the complex was not close enough to be cross-linked to the lipids, indicating that the binding order also reflected the order of protein layers on the surface of the forming vesicle.

1.2.3 Interactions between the coat forming proteins

The interactions between Sec23/24p, Sar1p, Sec13/31p and Sec16p elucidated the coat structure and a possible formation mechanism further. The Sec24p N-terminal half is responsible for Sec23p binding. Both the N- and the C-terminal domains of Sec24p are bound to the central domain (residues 565-1235) of Sec16p. Sec23p is attached to the C-terminal part (residues 1638-2194) of Sec16p (Shaywitch *et al.* 1997). Sec16p also binds to the middle region of the C-terminal portion of Sec31p (Shaywitch *et al.* 1997). The N-terminal domain of Sec31p is responsible for binding to Sec13p and the central domain to Sec23/24p. In *in vitro* studies with synthetic liposomes and nonhydrolyzable GTP analogue, Sec16p was not needed, but *in vivo* it was essential for vesicle budding. It seems that Sec16p serves as a platform for the assembly of the coat with interactions to other coat components.

1.2.4 Kinetic studies

In light scattering studies, mixing purified COPII proteins with liposomes altered the fluorescence amplitude, as proteins polymerized onto the liposome surface. From fluorescent changes Antony and coworkers (2001) noticed that in the presence of Sar1p preloaded with GTP, the Sec23/24p complex bound instantly to lipids. The complex also dissociated from the liposomes due to the GAP-activity of Sec23p, with a halftime of about 30 seconds. Adding Sec13/31p to the mixture led to the dissociation of the complex with a halftime of a few seconds, indicating that the Sec23p GAP-activity is stimulated by Sec13/31p binding. *In vitro* empty vesicles without cargo proteins budded from the liposomes as well as from cargo-depleted ER membranes, if the nonhydrolyzable GTP analogue was used (Matsuoka *et al.* 1998). In the presence of GTP and the coat components Sar1p, Sec23/24p and Sec13/31p, vesicles are not formed due

to instability of coat polymerization due to inborn GAP-activity. Adding the cytoplasmic catalytic domain of Sec12p to the reaction mixture supported budding profile formation, as if continuous loading of Sar1p-GTP was capable of preserving coat interactions (Futai *et al.* 2004).

1.2.5 Structural studies

1.2.5.1 Electron microscopy

Using a deep-etch rotary shadow technique, where platinum replicas were made as EM samples, Matsuoka and coworkers (2001) studied the structures of purified COPII components. The Sec23/24p complex turned out to be shaped like a bow tie. Sec13/31p complexes were flexible, probably heterotetramers, with a central rod and terminal globular structures. Structures of the same kind were also revealed with three-dimensional reconstruction from electron microscopic images of uranyl-formate stained purified complexes (Lederkremer *et al.* 2001). The bow tie structure of the Sec23/24p complex was a heterodimer composed of two similarly folded peptides of Sec23p and Sec24p, although these peptides share only low sequence similarity (Lederkremer *et al.* 2001). For the Sec13/31p, the reconstruction suggested five globular domains in a 30 nm long flexible rod. Gel filtration studies suggested that Sec13/31p is a heterotetrameric complex with a peptide ratio of 1:1. After crosslinking, a 220 kD heterodimer was isolated from a 700 kD complex. Sec13p alone is a globular 30 kD protein with seven WD40 propeller fold (Garcia-Higuera *et al.* 1998). Sec31p also has a WD40-like fold in the N-terminus, and the rest of the protein might fold like two separate α solenoid structures (Devos *et al.* 2004, see Gurkan *et al.* 2006, Fig. 9 D).

1.2.5.2 Crystallography

The bow tie-like, flat structure was confirmed for Sec23/24p with a crystallographic analysis of the yeast prebudding complex, which consisted of the Sec23/24p heterodimer with Sar1p bound to the nonhydrolyzable GTP-analogue. The structure was 15 nm long; it curved on the membrane side and contained positively charged side chains facing the membrane. Both Sec proteins had five different folding domains, a β-barrel, a zinc finger, a trunk domain, an α helical domain and a gelsolin domain. All four first mentioned domains partially formed the inner surface of the membrane contact area. The trunk domain also formed the contact area for the interactions within the dimer. The Sar1p binding site in Sec23p was formed partially from three domains, trunck, gelsolin and α helical domain (Bi *et al.* 2002).

Also the crystallographic structure of Sar1p in its active state was analyzed and compared with the inactive GDP-state

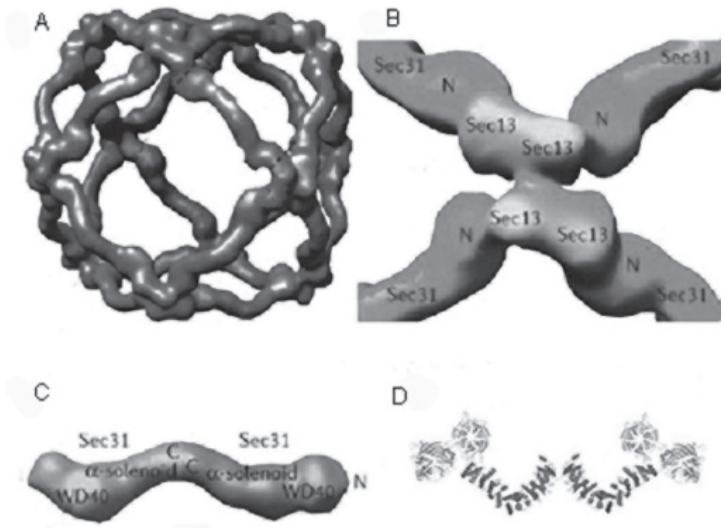

Figure 9. A) cuboctahedron structure of the COPII outer layer, B) Sec13p joints, C) the heterotetramer Sec13/31p complex, D) alpha solenoids and WD40 repeat folds of Sec13/31p complex. Reprinted by permission from Macmillan Publishers Ltd: Nature reviews Molecular Cell Biology Gurkan *et al*. 2006, Copyright 2006, Nature publishing Group

structure of the mammalian homolog (Bi *et al.* 2002). Mammalian Sar1p was shown to expose nine rather hydrophobic amino acids in the N-terminal conserved domain that promotes the membrane association to facilitate GDP exchange by Sec12p (Fig. 7). If a phenylalanine was changed to an asparagine residue in this sequence, Sar1p binding to membranes was lost, as was vesicle formation (Huang *et al.* 2001). The tight membrane association of Sar1p was achieved by a conformational change triggered by GTP binding that exposed an N-terminal amphipathic α helix. The helix inserted itself into a lipid bilayer and bent or curved the membrane into a tubule-like form (Bi *et al.* 2002, Lee *et al.* 2005). If bulky and hydrophobic amino acids of the amphipathic α helix were changed to alanine, cargo insertion into the forming bud and coat complex formation seemed to operate in *in vitro* assays with isolated ER membranes, but vesicle formation *in vivo* was lost (Lee *et al.* 2005). This indicates that the active Sar1p-GTP induced membrane bending is important for membrane fission. The crystal structure of the prebudding complex shed light also to the mechanism by which Sec23p can increase GTPase activity. Sec23p linked an arginine side chain (R722) into the Sar1p active site to speed up the reaction (Bi *et al.* 2002).

1.2.5.3 In vitro *studies of self-assembly of the COPII coat*

Previously, it was reported that vesicles coated with polymerized COPII, produced *in vitro* in the presence of a nonhydrolyzable GTP analogue from liposomes, contained equal amounts of the structural components (Barlowe *et al.* 1994, Antonny *et al.* 2001). Antonny and coworkers (2001) were also able to get spherical particles from liposomes in the presence of purified Sec23/24p and Sec13/31p in equimolar ratio.

Stagg and coworkers (2006) using cryo-EM at 30Å resolution were able to show that only the Sec13/31p complexes were able to form a cage from a flexible lattice, triangles and squares yielding a round structure which they called “cuboctahedron” (Fig. 9A). This indicates that the inborn forces of Sec13/31p polypeptides sculpt membranes with the aid of membrane anchors (e.g. Sec23/24p). In this structure, the formation of edges that connect the structural elements seemed to be created from four interacting Sec13 molecules (Fig. 9B), and filaments formed mainly through two Sec31p molecules (Fig. 9C). One unit was a Sec13/31p heterotetramer that is supposed to be formed by joining two Sec31p molecules via the C-terminus with the N-terminal WD40 domain binding to Sec13p (Stagg *et al*. 2006, see Gurkan *et al.* 2006).

1.2.6 Recognition of cargo by COPII coat components

Many different types of signatures are found in the cytoplasmic tails of exocytic transmembrane cargo or putative soluble protein receptors; recycling proteins that aid cargo packaging into COPII coated vesicles. Those signatures might be folds or short sequences like di-hydrophobic, di-acidic or di-basic motifs (see Bonifacino and Glick 2004). Divergent binding motifs require several acceptor binding sites. Most of the binding sites known to date are located on the membrane-lining surface of Sec24p (Mossessova *et al.* 2003, Miller *et al.* 2003), but also Sar1p and Sec13/31p binding may have an influence (e.g. Campbell and Schekman 1997, Springer and Schekman 1998, Belden and Barlowe 2001a).

1.2.6.1 SNAREs

When vesicles are formed *in vivo* they should contain both transported cargo and address information for where they are targeted, in order to maintain correct transport direction and the specificity for a certain acceptor membrane compartment. SNAREs were identified as coiled coil membrane associated proteins that at a fusion event form a tight protein interaction “zipper” bringing two separate membranes close to each other leading to fusion. Target membranes contain an assembled t-SNARE complex and vesicles a v-SNARE which together provoke membrane fusion. Only correct SNAREs are fusogenic and they yield specificity to the fusion event, although some SNAREs are able to function at several steps (e.g. Tsui and Banfield 2000, Liu and Barlowe 2002, see Hong 2005).

Transport vesicles formed from the ER membrane loaded with exocytic cargo contain Bet1p as a v-SNARE. An assembled t-SNARE complex of Sed5p, Sec22p and Bos1p, is also present on the vesicle (Newman *et al.* 1992, Rexach *et al.* 1994, Mossessova *et al.* 2003). Using a glutathione S-transferase (GST)-fused cytoplasmic domain of Sed5p, a direct interaction with Sec24p was seen (Peng *et al.* 1999). Bet1p and Bos1p were shown to interact with a prebudding complex in a Sar1p-GTP-dependent manner (with nonhydrolyzable GTP analogue; Springer and Schekman 1998). Mossessova and coworkers (2003) were able to show that the v-Bet1p and t-SNAREs Sed5p and Sec22p bound through direct contacts with the Sec23/24p complex. From Bet1p, they mapped a well-conserved LXXLE-motif (LXX-L/M-E) that allowed Bet1p alone to bind to the so called B-site of Sec24p (Fig. 8). The same motif was found also in Sed5p, as well as an YNNSNPF motif. The second motif bound to a different area of the Sec24p, A-site (Fig. 8). Furthermore, binding to the YNNSNPF motif depended on the state of the t-SNARE complex. This sequence was exposed only after SNARE complex formation.

Binding to the hydrophobic A-site seemed to be more based on a folding pattern than a specific amino acid sequence, since the motif was not conserved through evolution. Mutating tryptophan 897 in the A-site to alanine impaired binding of Sed5p to Sec24p, whereas other SNAREs were uninfluenced (Miller *et al.* 2005). Vesicles containing the Sec24pW897A mutant were not able to fuse with Golgi membranes, although Sed5p immuno-depleted vesicles were. This suggests the A-site to be an important binding site also for an unknown factor needed for fusion. The binding site for Sec22p in the Sec24/23p interface (C-site, Miller *et al.* 2003) seemed to be a folding structure, based on crystal structure of Sec22p bound to Sec23/24p complex. In this structure Sec22p bends and forms a closed structure with its N-terminal longin domain and NIE-segment leading to single unassembled SNARE packaging. In the assembled SNARE complex, the NIE-segment is masked (Mancias and Goldberg 2007).

1.2.6.2 Other cargo molecules

The first indication of the involvement of the Emp24p protein in ER to Golgi transport came from an *EMP24* deletion strain where some proteins (invertase and Gas1p) were transported with slower kinetics than in normal cells, and the retention of ER-resident proteins was impaired. Deletion of *EMP24* also suppressed *SEC13* deletion, which alone is lethal (Schimmoller *et al.* 1995, Elrod-Erickson and Kaiser 1996). Deletion of another gene, *ERV25,* resulted in a similar secretion phenotype as deletion of *EMP24*. Furthermore, Emp24p and Erv25p were incorporated into COPII-coated vesicles only as a heterocomplex, and both contained a di-phenylalanine hydrophobic sequence in their cytoplasmic tail, responsible for COPII binding. A synthetic decapeptide of the C-terminal tail of Emp24p and Erv25p bound to sepharose beads discriminated between Sar1p binding: only the Emp24-tail bound to it directly. Both Sec23/24p and Sec13/31p complexes seemed to bind to C-tail peptides *in vitro*. In titration experiments Sec13/31p had stronger affinity to C-tails than Sec23/24p (Belden and Barlowe 1996, Belden and Barlowe 2001a). Emp24p and Erv25p are directly bound to the GPI-linked cargo protein Gas1p on isolated transport vesicles, suggesting a receptor-like function for Emp24p and Erv25p (Muniz *et al.* 2000). Both belong to the nonessential yeast protein family of 8 members (p24-family; Emp24, Erv25, Erp1-6) that are packaged into COPII-coated vesicles, and cycle between the ER and the Golgi (Marzioch *et al.* 1999, Springer *et al.* 2000). Since they form a large heterocomplex, they may be important for an exclusion mechanism in cargo selection (Marzioch *et al.* 1999, Emery *et al.* 2003). In mammalian cells defective in retrotransport from the Golgi to the ER, p24-family members were misplaced, and formed large oligomers in endomembranes that segregated away from cholesterol rafts. Emery and coworkers (2003) suggested the role of the p24 family to be exclusion of cholesterol from the membranes of transport vesicles. However, the exact role

of p24 members in COPII formation is at the moment elusive.

Another nonessential transmembrane protein, Erv14p, which recycled between the ER and the Golgi was needed for proper targeting of the membrane protein Axl1p. Erv14p was shown to bind to Axl1p and interact with COPII coat via its cytoplasmic loop (Powers and Barlowe 1998, Powers and Barlowe 2002). Otte and coworkers (2001) identified by mass spectrometry from purified COPII vesicles nine abundant proteins, Erp1p, Erp2p, Erv29p, Erv41p, Erv46p, Emp47p, Rer1p, Yip1p and Yif1p that had not been found in the COPII vesicles before. Erv29p was later shown to be responsible for packaging of pro-α factor, a soluble cargo protein, into the COPII coated vesicles. Transport of another soluble protein carboxypeptidase Y (CPY), was delayed in an *ERV29* deletion strain (Belden and Barlowe 2001b). A hydrophobic interrupted signature (I-L-V) in the pro-region of α factor functioned as a dominant, saturable determinant, needed for binding to Erv29p and for packaging into COPII vesicles, since it also directed the export of a chimeric ER-resident protein (Otte and Barlowe 2004). The binding motif of Erv29p to the COPII complex is unresolved. From purified COPII-coated vesicles also a 26 kD protein was isolated and named Erv26p. Its deletion resulted in impaired transport of the precursor of alkaline phosphatase (Bue *et al.* 2006).

A di-acidic binding signature (DxE) was found in an integral membrane protein Sys1p. Its cytoplasmic tail appeared to be responsible for insertion of Sys1p into COPII vesicle membranes (Votsmeier and Gallwitz 2001). The DxE motif bound to a conserved positively charged cavity, overlaid with the binding site LXXLE (B-site) in Sec24p (Miller *et al.* 2003, Mossessova *et al*. 2003). There evidently are new interacting sites to be discovered. The same site may be used for recognition of different proteins. Sec22p, Bos1p, Erp1p, Erp2p and Emp24p packaging was reduced by mutation of the Bet1p/Sys1p binding site (Miller *et al.* 2003).

Some transport signatures were sequential transport signals. Transmembrane proteins Erv41p and Erv46p formed a pair in the ER lumen and both proteins were needed for the complex transportation by exposing signatures in their C-terminal tails. An isoleucine–leucine signal in one tail bound COPII and a second phenylalanine-tyrosine motif in the other tail ensured packaging to COPII vesicle (Otte and Barlowe 2002). Another protein pair is formed between Emp46p and Emp47p, proteins that have homology with lectins in their luminal domains, implicating a putative glycoprotein receptor property. Transport of Emp46p relied on the formation of a hetero-oligomeric complex with Emp47p. The complex between Emp46p and Emp47p formed through coiled coil domains in the ER lumen, followed by insertion of the proteins into COPII vesicles with the aid of C-terminal cytoplasmic hydrophobic sorting sequences (Sato and Nakano 2002 and 2003).

Oligomer formation seems to be a common theme to ensure efficient packaging into COPII-coated vesicles, as Sato and Nakano (2004) were able to show for Emp46/47p. They used cargo loading into neutral proteoliposomes; Emp47p or the coiled-coil deletion mutant version of Emp47p (Emp47-281-333) in combination with Emp46p and an ER resident SNARE Ufe1p. Sec23p and Sec31p binding to membrane was dependent on the C-terminal tail of Emp47p. Both the wild type and the deletion version were shown to bind COPII components equally. The difference was in the incorporation into COPII coated vesicles: only the wild type Emp47/46p-complex was efficiently incorporated, indicating a cargo oligomerization requirement for the engulfment into vesicles.

Specific cargo substrates for these lectin-like putative receptors have not been identified yet, although reduction of high mannose containing glycoprotein transport to culture medium was reported (Sato and Nakano 2002). The mammalian homolog, ERGIC-53, binds to the lysosomal glycoprotein cathepsin Z, and mutations in ERGIC-53 led to a bleeding disorder due to transport deficiency of blood coagulation factors V and VIII (Appenzeller-Herzog et *al.* 2005, Nichols *et al.* 1998). Furthermore,

Appenzeller-Herzog and coworkers (2005) suggested cargo binding to be dependent not only on the carbohydrate, but also on a β-hairpin structure on the glycoprotein. Thus, the protein folding recognition dependent binding to the glycan is part of the ER quality control machinery. If the protein is not correctly folded locally, it is not recognized for transport.

A subset of cargo molecules might be recruited through interactions with GTP-loaded (GMP–PNP) Sar1p, as demonstrated in Sar1p-GST pull-down experiments. Emp24p, Hip1p and Sec22p bound to Sar1p, the efficiencies being enhanced by Sec23/24p complex addition (Kuehn *et al.* 1998). The SNAREs Bet1p and Bos1p associated with Sar1p even without GTP (Springer and Schekman 1998). In mammalian cells, another type of mechanism, functioning in packaging of Golgi-resident glycosyltransferase, required a di-basic export motif, [RK](X)[RK], and direct binding to Sar1p (Giraudo and Maccioni 2003). This kind of a motif has not been shown to function in yeast, although a similar sequence KRRIR is present for example in the cytoplasmic N-terminus of N-acetylglucosaminyltransferase 1 (Gnt1p) (*Saccharomyces* genome database; http://www.yeastgenome.org/).

1.2.7 Variation in coat composition

Two nonessential Sec24p homologs, Sec24Bp (56% similarity) and Sec24Cp (23% similarity), have been found in the yeast genome. Functional homology has been shown by their ability to bind Sec23p and Sed5p (Pagano *et al.* 1999, Peng *et al.* 2000). Deletion of *SEC24C* affected the ER exit of the plasma membrane ATPase (Pma1p) and was synthetically lethal with *sec13-1* (Roberg *et al.* 1997). Pagano and coworkers (1999) showed that a subset of soluble secretory proteins was affected at 37ºC by deletion of *SEC24C*. Overexpression of Sec24Bp supported the growth of *SEC24* deletion strain, while overexpression of Sec24Cp did not (Higashio *et al.* 2000, Kurihara *et al.* 2000). In *in vitro* studies Sec24Bp formed vesicles in combination with Sec23p and Sec13/31p like Sec24p. Deletion of *SEC24B* was lethal in a SNARE mutant *sec22-3* background indicating a functional role for Sec24Bp *in vivo* (Kurihara *et al.* 2000). Miller and coworkers (2003) showed that Sec24Cp deficient extracts could generate vesicles *in vitro*. Those vesicles, however, lacked Sec22p, Bet1p, Bos1p, Sed5p and Emp24p, as well as the cargo protein Gap1p, but included cargos Gas1p and pro-α factor. Furthermore, vesicles were unable to fuse with receptor membrane. Sec24Cp must function in combination with other Sec24 family proteins in order to produce functional vesicles *in vivo*. The exact role of Sec24Bp is obscure.

1.2.8 Polymerization of the COPII coat

First, cytoplasmic Sar1p-GDP associates via its N-terminal helix to ER membranes and collides by lateral diffusion to the GDP-exchange factor Sec12p that facilitates GDP release, whereafter Sar1p is ready to bind GTP. GTP-binding induces a conformational change that exposes a binding site for Sec23p as well as an N-terminal amphipathic α helix that inserts firmly into a lipid layer (Fig. 7). The helix probably displaces lipid head groups on the surface of the ER membrane, and forces the membrane to bend. Sec12p covers ER membranes uniformly, but Sec13p and Sec16p appear to occur in foci, an indication of budding sites (Rossanese *et al.* 1999). Activated Sar1p then recruits Sec23/24p, leading to so called prebudding complex. The nucleation of the prebudding complex and coat polymerization is also influenced by other protein factors like Sec16p. Sec16p promotes with Sar1p-GTP the polymerization of the COPII coat by stabilizing the coat through several interactions with the other coat proteins (Supek *et al.* 2002).

The cargo is harvested by the prebudding complex mainly through Sec24p interactions with cytoplasmic motifs of integral membrane proteins and Sar1p cargo interactions. The prebudding complex formation also includes its dissociation, since degradation of Sar1p-bound GTP is accelerated by GTPase activating protein Sec23p. Cargo binding also accelerates GTPase leading to complex dissociation (Kuehn *et al.* 1998, Yoshihisa *et al.* 1993,

Antonny *et al.* 2001, Sato and Nakano 2004). GTPase activity is proposed to act as a cargo sorting device. *In vitro* produced vesicles from proteoliposomes differed between cargo exclusion in the presence of GTP. Without GTP hydrolysis wild type Emp46/47p complex and monomeric Emp47p were included into vesicles with the same kinetics (Sato and Nakano 2004). In fluorescence resonance energy transfer (FRET)-based experiments on proteoliposomes Sato and Nakano (2005) showed that the correct cargo associated with the Sec23/24p complex for many GTP-GDP cycles if the cytoplasmic domain of the GTP exchange factor Sec12p was included in the reaction. Correct SNAREs are able to keep the association with the Sec23/24p-complex during Sar1p cycling and this might be based on multiple binding signals and oligomerization. Altogether these *in vitro* budding assays on cargo loaded proteoliposomes suggested that the correct cargo binding delayed Sec23/24p dissociation giving time to more stable coat polymerization, and since any cargo binding accelerated the GTPase activity leading to incorrectly bound cargo dissociation, GTPase cycle is a very important cargo selection mechanism.

As already mentioned, Sar1p-bound Sec23/24p complex recruits Sec13/31p complex that has interactions to both Sec23p and Sec24p as well as to Sec16p. The binding of Sec13/31p complex accelerates GTPase by a factor of ten, which leads to Sar1p dissociation from the membrane. Outer coat structure presence stabilizes coat protein interactions, which are needed for coat polymerization, membrane deformation and vesicle fission. The prebudding complex formation with correct cargo binding keeps Sec23/24p on the membrane long enough that it is polymerized in the forming bud with interactions to the lattice forming components Sec13/31p (Fig. 10). Curvature to the membrane is initiated by the Sar1p amphipathic helix. The bent form of the membrane fits well the concave structure of Sec23/24p and is fixed with the outer core of the lining coat Sec13/

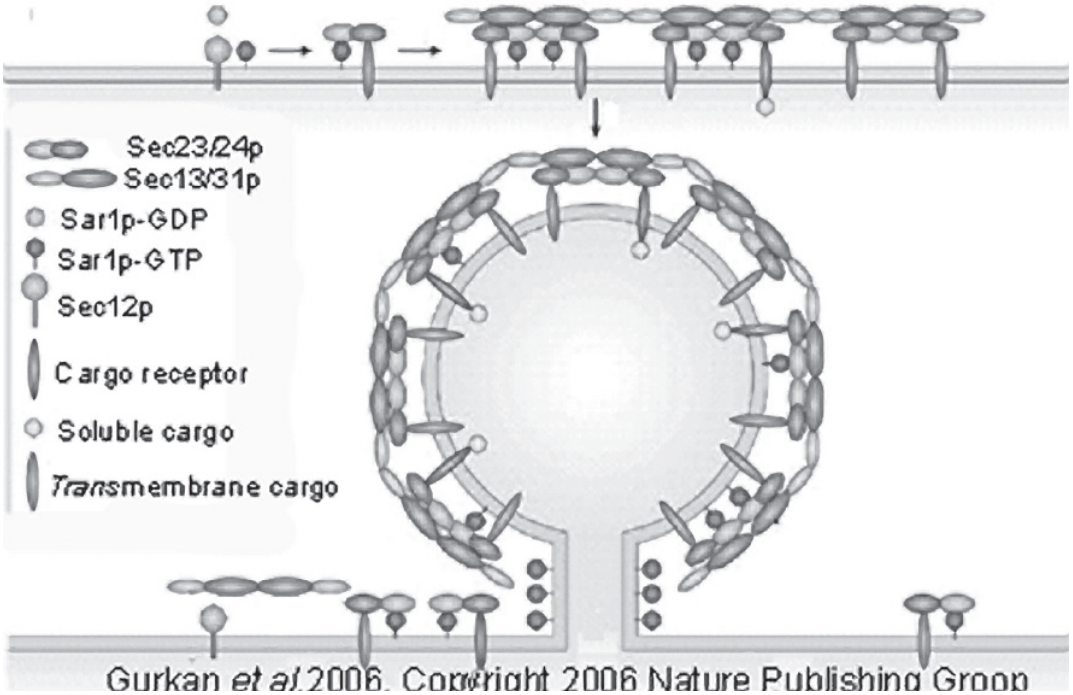

Figure 10. A schematic picture of events leading to COPII coat polymerization and coated vesicle fission. Reprinted by permission from Macmillan Publishers Ltd: Nature reviews Molecular Cell Biology Gurkan *et al*. 2006, Copyright 2006, Nature publishing Group.

31p that has an endogenous capacity for forming a spherical cage. Sec12p-charged new Sar1p-GTP molecules at the boundary between a bud and the ER exit site maintain an interaction with Sec23/24p as long as they are not incorporated to the “permanent” cage by binding to Sec13/31p. Cycling and dissociation is kept going by the GTPase cycle, and the coat polymerization is more likely to happen with oligomeric stable cargo interactions than opportunist single, weak, short-lived interactions. After vesicle fission at some point the coat depolymerises and the vesicle is ready for homo or heterotypic fusions to the next compartment (reviewed Hughes and Stephens 2008).

1.3 Golgi apparatus

Cammillo Golgi in his histological studies of neuronal cells in the 1890s discovered a structure that he designated as “internal reticular apparatus”. Thereafter the organelle was named the Golgi apparatus. Its existence was accepted after the progress of fixation of EM-samples in the 1950s. The main role of the Golgi is to sort, modify and deliver the secretory cargo to its correct destinations. In mammalian cells it is formed of stacks of membrane-enclosed compartments called cisternae. The subcompartments have partially different enzyme compositions. The subcompartments are named *cis*, *medial* and *trans* Golgi, from the protein entry side to the exit side.

How proteins are sorted inside the Golgi complex is under an ongoing debate. There is evidence for and against both main theories “Cisternal maturation” and “Static compartment” (Glick and Malhotra 1998, Pelham and Rothman 2000, Rabouille and Klumperman 2005). In the static compartment theory, Golgi compartments are pre-existing and cargo is transported from one cisterna to another. According to the cisternal maturation theory new cisternae are generated from the *cis* face of the stack, followed by their development into *medial* and *trans* cisternae by retrotransport flow of Golgi glycosylation enzymes. At the *trans* face of the Golgi, the last cisterna is spent to form forward cargo vesicles, as well as Golgi enzyme retrotransporters. A third Golgi traversing system was proposed from electron tomography studies of mammalian cells: a synchronized traffic wave was suggested that induced the formation of inter-cisternal connecting tubules along which cargo as well Golgi enzymes were distributed (Trucco *et al*. 2004).

The mammalian-like Golgi stacks where olisaccarides are processed can be seen in yeasts like *P. pastoris* and *Schizosaccaromyces pombe*, but not in wild type *S. cerevisiae* cells (Mogelsvang *et al.* 2003, Ayscough *et al.* 1993, Preuss *et al.* 1992). *S. cerevisiae* mutants *sec7* and *sec14* accumulate at restrictive temperature structures that resemble Golgi stacks (Novik *et al.* 1981). The cup shaped, extended cisternae were identified and named Berkeley bodies. Preuss and coworkers (1992) studied with immuno-EM normal *S. cerevisiae* cells and discovered several disk-like structures that contained Golgi markers and were surrounded by vesicles, apparently fragmented Golgi elements.

Graham and Emr (1991) studied the transport of α-factor and CPY in the temperature sensitive secretory mutant *sec18-1,* where vesicular fusions are prevented due to the failure to resolve assembled SNAREs after membrane fusion on a target membrane (Grote *et al.* 2000). In the rapidly inactivated *sec18-1* mutant they found deficiency in the procession of at least three different types of protein modifications in protein intermediates produced at permissive temperature. A portion was in the ER form, but proteins with α1-6-elongated N-glycans, and α1,3-mannosylated but not Kex2-protease processed intermediates were resolved. The sequential deficiency in modifications suggested that enzymes locate in different compartments requiring functional vesicular transport between compartments. In the *sec23-1* strain in a similar experiment, ER forms were not able to mature at restrictive temperatures. This was an indirect proof of yeast Golgi complex compartmentalization.

In EM studies of *sec18-1* cells at restrictive temperature, vesicle formation was synchronized to study the disappearance and reappearance of

organelles that transformed into secretion vesicles. In wild type cells vesicles were rarely encountered, but at the restrictive temperature in the *sec18-1* strain vesicles accumulated and nodular networks, presumably Golgi structures, disappeared. After releasing the block, vesicles bridged with membrane connections, and nodular networks reappeared. The nodular size first resembled the size of transport vesicles. Later, membrane connections between large nodules got thinner and finally they broke releasing secretory vesicles to the cytoplasm (Morin-Ganet *et al.* 2000). Rambourg and coworkers (2001) used a retrotransport (*sec21-3*) mutant, and observed the accumulation of ribbon-like elements. After releasing the transport block they noticed the accumulation of glycosylated material in the intersections of the ribbons but no formation of separate vesicles.

These studies suggest that the yeast Golgi is composed of scattered elements all over the cytoplasm. They appear as membrane processions when the secretory material exits the ER, and active SNARE dependent fusions between membrane compartments are needed for protein intermediates to mature.

In mammalian cells the Golgi is one membrane complex, close to the nucleus. The secretory protein containing organelle has to travel a long distance along microtubules to reach the Golgi. This transport happens in membrane containers that are formed close to ER exit sites, so called vesicular tubular cluster (VTC) areas (Saraste and Kuismanen 1984, see Mogelsvang *et al.* 2004). The existence of VTCs in yeast is not obvious. Homotypic vesicle fusion seen after release of the *sec18-1* secretion block might represent yeast VTC as observed by Morin-Garnet and coworkers (2000).

Matsuura-Tokita and coworkers (2006) followed by three-dimensional confocal microscopy *in vivo* the yeast Golgi using fluorescent markers fused to the proteins of the early and late Golgi. They saw membrane structure contents slowly progressing from the *cis* to the *trans* direction, supporting the cisternal maturation theory. Another group studied the kinetics of the cisternal maturation also by three-dimensional time-lapse fluorescence microscopy. They proved the same maturation of the early Golgi marker (Vrg4p GFP-fusion) to the late Golgi marker (Sec7p fusion to DsRed) in individual cisternae with a consistent rate. In half time of three minutes, a visible cis Golgi marker in an individual cisterna was progressively changed to the late Golgi marker and then the late Golgi signals fainted away also with the halftime of three minutes. Furthermore, they could link the Golgi transition to the time in which the secretory cargo, α factor and CPY, progressed through the Golgi (the halftime of seven minutes). The protein transport rate also supported the cisternal maturation of the yeast Golgi (Losev *et al.* 2006).

1.3.1 Vesicle fusion and essential factors

At the moment it is not totally clear what happens after pinching off of the vesicle from the ER membrane. It is still debated whether vesicles are formed *in vivo*, or whether the cargo departs from the ER in large containers without vesicle formation (Mironov *et al.* 2003). However, real vesicles have been isolated from yeast cells and these vesicles are able to fuse with Golgi membranes *in vitro,* based on glycosylation studies (Rexach and Shekman 1991). Factors like Ypt1p, Sec19p, Sly1p, Sec7p and Uso1p have been found to be necessary for fusion (Lupashin *et al.* 1996, Franzusoff *et al.* 1992). SNAREs guarantee proper membrane fusion, but before SNARE action, compartment-specific tethering machineries mediate the initial pairing of the membranes.

In vitro studies with washed membranes and purified COPII proteins demonstrated vesicle attachment (docking) to membranes before the actual fusion event, triggered by addition of the purified peripheral membrane protein Uso1p and energy. Vesicle attachment to the Golgi was prevented by adding Sec19p-GDP dissociation inhibitor (GDI) that extracted Ypt1p from membranes (Cao *et al.* 1998). Ypt1p is an essential member of Rab-GTPases that mediates Uso1p-binding. GDI extraction is part of the

normal Rab-cycle, where Rab-GDP proteins that harbour dual prenylation (Callero *et al.* 2003) are removed from receptor membranes to recycle back to the donor membrane, such as a forming or formed vesicle at the ER membrane. Without being escorted by GDI, the hydrophobic prenyl tails would aggregate in the cytosol.

How protein tethering is regulated is unknown, but a multisubunit, exocyst-like tethering complex TRAPP I is reported to be responsible for GEF activity towards Ypt1p, suggesting that the TRAPP I complex may mark places where at least the activation of Ypt1p to the GTP-bound form happens (Jones *et al.* 2000). TRAPP I complex seemed to be directed on the vesicle by interaction with Sec23p (Cai *et al.* 2007). There are also reports of Ypt1p and Uso1p participation in the sorting events of ER exit of the GPI-linked proteins and likewise, a large multimeric protein complex, COG has been suggested to participate in protein sorting from the ER (Morsomme and Riezman 2002). The COG-complex was earlier reported to be required for the early secretion pathway and then for the tethering of vesicles in retrotransport (Wuestehube *et al.* 1996, Suvorova *et al.* 2001, Whyte and Munro 2001).

When vesicles are formed at the ER exit sites, the polymerizing coat prefers the vesicular SNARE Bet1p alone and on the other hand has affinity towards the assembled t-SNARE complex. This leads to a situation where homotypic vesicle fusion is possibly assisted by the ER to Golgi v-SNARE Bet1p and the Golgi target SNARE complex Sed5p, Sec22p and Bos1p. Also a direct vesicle fusion to the *cis* Golgi with the vesicular v-SNARE Bet1p and at the target membrane residing t-SNARE complex could happen. The third possibility is a fusion of the anterograde vesicle with a retrotransport vesicle containing the *cis*-Golgi transferases that leads to formation of a new *cis*-Golgi. *In vitro* with mammalian cells, the homotypic COPII vesicle fusion was shown to happen in formation of a new VTC compartment (Xu and Hay 2004). SNAREs are also regulated by phosphorylation (Weinberger *et al.* 2005). A typical protein kinase A (PKA) phosphorylation site close to the *trans*-membrane domain was shown to affect the ER-Golgi transport. Upon expression of a non-phosphorylated mutant (an alanine substitution) of Sed5p, the mammalian-like Golgi structures appeared in yeast. An aspartate substitution that mimics the phosphorylated state accumulated the ER and transport vesicles and reduced growth.

1.4 Recycling

After vesicle fusion, membranes and contents mix, and the soluble proteins are released from their receptors. The release may be due to a pH difference. The mammalian lectin ERGIC-53 binds to mannose at pH 7.4 but not in the presence of low calcium at pH 6.5 and below (Appenzeller-Herzog *et al.* 2004). The release is evident, since receptors and putative receptors (Emp-family) are not detected with the cargo in later secretory compartments; instead, they are recycled back to the ER for another round of binding. The recycling of the heterodimeric Emp24/Erv25p complex back to the ER is dependent on a cytoplasmic dilysine sequence (KKXX) in the Erv25p C-terminus (Belden and Barlowe 2001a). The putative cargo receptors Emp46/47p dissociate from each other in the Golgi (Sato and Nakano 2003, Eugster *et al.* 2004). Also SNARE proteins are recycled back to the ER (Ballensiefen *et al.* 1998, Ossipov *et al.* 1999, Lewis *et al*. 2004).

Many Golgi-resident proteins like glycosyltransferases slowly recycle between the ER and the Golgi (Todorow *et al.* 2000, Karhinen and Makarow 2004). Another type of recycling is retrieval of the proteins escaped from the ER, like Kar2p containing in the C-terminus the HDEL (KDEL in mammals) sequence (Munro and Pelham 1987). HDEL is recognized in the Golgi by the receptor Erd2p (Semenza *et al.* 1990), which directs HDEL-containing proteins back to the ER. The same retrieval system is used by toxins like cholera-toxin (Majoul *et al.* 1996). Some transmembrane proteins do not contain obvious retrieval sequences, but are returned due to recognition by another transmembrane protein (Rer1p) through a transmembrane hydrophobic sequence

flanked with polar residues (Boehm *et al.* 1997, Sato *et al.* 1997, Sato and Nakano 2003). Recycling back to the ER from the *cis*-Golgi is dependent on retrotransport mediated by COPI-coated vesicles.

1.5 COPI

1.5.1 Identification of COPI-coated vesicles

The first indication of COPI coated vesicles came from isolated mammalian Golgi membranes that produced vesicles, when cytosol and energy were added. These vesicles were different from clathrin-coated vesicles by morphology, and clathrin antibodies did not recognize them (Orci *et al.* 1986). A coatomer (coat protomer), the putative cytosolic coat structure (700 kD) was found to consist of seven protein subunits. The largest proteins were named α-, β-, γ- and δ-COP (Waters *et al.* 1991). The coatomer was named COPI (Barlowe *et al.* 1994). A temperature-sensitive vesicular stomatitis virus glycoprotein (VSV-G) mutant can be accumulated at restrictive temperature in the ER and released for transport by lowering the temperature. Its transport could be blocked also by microinjecting antibodies raised against the β-COP protein (Pepperkok *et al.* 1993). Transport was blocked to the ER and the VTC compartment but not to the *trans*-Golgi. This suggested that COPI operated at the early stages of the secretory pathway.

1.5.2 Identification of yeast COPI components

Proof for COPI components in yeast came from the identification of an indispensable yeast protein, Sec21p, as a component of a cytosolic complex similar to the mammalian COPI coatomer (Hosobuchi *et al.* 1992). Stenbeck and coworkers (1992) recognized that a portion of a mammalian γ-COP gene was homologous to the yeast *SEC21* gene. The yeast β-COP gene was cloned, based on mammalian β-COP antiserum recognizing isolated yeast coatomer proteins. The corresponding protein was trypsinized to peptides, which were sequenced, and the equivalent gene was isolated from a gene library by DNA probes based on the peptide sequences. Another large coatomer protein was cloned with the same strategy. These genes were named *SEC26* (β-COP) and *SEC27* (β'-COP) (Duden *et al.* 1994).

Some type I membrane proteins in mammalian cells harbored a signature in their C-terminal cytoplasmic domain (di-lysine; KKXX) that was responsible for ER location (Nilsson *et al.* 1989). This signature joined to heterologous proteins also located those proteins to the ER, however, they were Golgi modified indicating transport to the Golgi followed by retrieval back to the ER (Jackson *et al.* 1993). Retrieval of proteins harbouring the KKXX-motif to the ER was shown to happen in yeast cells as well (Gaynor *et al.* 1994). A yeast α-COP homolog was identified in a screen of membrane proteins harbouring the KKXX motif (Letourneur *et al*. 1994). The screen was based on a chimeric protein, a plasmamembrane-located α factor receptor protein fused to WBP1 (Ste2-WBP1) harbouring KKXX motif (in a *STE2* deletion strain), unsuccessful retrieval leading to plasma membrane localization and mating. Also *ret2-1* (δ-COP) and *ret3-1* (ζ-COP) were identified with a similar strategy, and cloned by complementation (Cosson *et al.* 1996). Yeast ε-COP homolog was found in a suppressor screen of the temperature-sensitive mutant *ret1-3,* and named *SEC28* (Duden *et al.* 1998).

1.5.3 ARF proteins

In addition to the cytoplasmic COPI coatomer, another component necessary for COPI polymerization *in vitro* is the ADP ribosylation factor (ARF). The ARF protein was identified as a cofactor in a reaction, where cholera toxin irreversibly activated adenylate cyclase by ADP ribosylation of a stimulatory subunit. The cofactor, ARF, was purified from rabbit liver membranes and turned out to be a 21 kD ubiquitous protein belonging to the Ras superfamily of GTP-binding proteins (Kahn and Gilman 1984).

Yeast has three homologous ARF proteins, of which Arf1p and Arf2p are 96% identical and function in the early secretion pathway. Arf3p seems to have a role in cell polarity development (Stearns *et al.* 1990, Lee *et al.* 1994, Huang *et al.* 2003). Deletion

of both *ARF1* and *ARF2* was lethal; *ARF1* deletion gave a cold-sensitive phenotype and the *ARF2* deletion strain behaved like normal cells (Stearns *et al.* 1990). The ARF proteins have several functions; one is serving as a promoter for coatomer assembly on Golgi membranes (see Rothman and Wieland 1996). Myristoylated ARF and GTP were needed for binding of the coatomer to isolated Golgi membranes (Palmer *et al.* 1993). As a member of the Ras family, ARF also cycles between a membrane-bound activated GTP-bound form, and a soluble, cytosolic form released by GTP hydrolysis (Peyroche *et al.* 1996). The main pool of ARF is GDP-bound and cytosolic. In the cytosolic form the myristoyl tail is shielded by the protein itself. The regulators of this cycle are again GEFs and GAPs. All Sec7 domain-harbouring proteins are putative activators of ARF. Yeast harbours four different potential GEFs and three of those localize to the ER-Golgi interface; Gea1p, Gea2p and Sec7p (see Jackson and Casanova 2000). Potential GAPs in the early secretory pathway are Gcs1p and Glo3p which harbour a zinc-finger motif (Poon *et al.* 1996, Dogic *et al.* 1999).

1.5.4 COPI coat in the early secretory pathway

In yeast mutants deficient in COPI components like Sec21p, anterograde transport was found to be defective (Hosobuchi *et al.* 1992). Sec26p-depleted cells also accumulated cargo proteins in the ER (Duden *et al.* 1994). Also in mammalian cells the anterograde block was evident, e.g. after injection of β-COP antibody (Pepperkok *et al.* 1993), or after expression of an ARF1(T31N)-mutant that is blocked in the GDP form (Dascher and Balch 1994). However, components of the yeast coatomer were found to operate in retrotransport from the Golgi to the ER (Letourneur *et al*. 1994, Cosson *et al.* 1996). Gaynor and Emr (1997) studied transport defects in the *sec21* mutants and concluded that COPI is required for retrotransport and that the defects in anterograde transport were secondary effects resulting from the Golgi to ER transport block.

As already discussed, in mammalian cells the Golgi is one complex close to the nucleus and VTC are elements that form close to ER exit sites. A cargo container travels along microtubular connections from the VTC to the Golgi area (Saraste and Svensson 1991, Scales *et al.* 1997). The COPI coat was found to be polymerised and remained stably attached onto the VTC and the pre-Golgi transport containers, but the signal from retrotransport vesicles was transient (Lippincott-Schwarz and Liu 2006). In the transport container, cargo segregated into an area different from the COPI-coated pole. No evidence of COPII was detected for this transport step (Stephens *et al.* 2000, Presley *et al.* 2002). At least in higher eukaryotes, COPI polymerization had two different roles in the early secretion pathway. In the Golgi, COPI vesicles might have additional roles in transport between endosomes and the Golgi and between Golgi subcompartments (see Rabouille and Klumperman 2005).

1.5.5 Structure of COPI coat

In vitro the COPI coat was disassembled in a high concentration of salt, and reassembled in physiological conditions; α-, β'- and ε-COPs interacted together, as well as γ- and ζ-COP (Lowe and Kreis 1995. 1996). Two-hybrid data suggested interaction between Sec21p (γ-COP) and Sec26p (β-COP). This interaction was detectable only in the presence of Ret3p (ζ-COP) (Takatsu *et al.* 2001). Sec27 (β'-COP) seemed to have in its conserved amino-terminal domain five WD-40 repeats and Ret1p (α-COP) six (Duden *et al.* 1994). Deletion of both WD40 domains together is lethal (Eugster *et al.* 2000, 2004).

In the γ-COP secondary structure predictions, the N-terminal α-helical domain and the C-terminal α-appendage resembled adaptin AP subunits, although the sequence similarity was extremely low. In X-ray crystallography the C-terminal part of γ-COP (764-874 amino acids) was solved with 2.3 Å resolution (Hoffman *et al.* 2003). It folded to a similar structure as the AP2 adaptor appendage domain that is important for protein recruitment onto clathrin-coated vesicles (Collins *et al.* 2002).

1.5.6 COPI polymerization *in vitro*

The minimal set of components to produce functional vesicles was studied *in vitro* by Ostermann *et al.* (1993). They showed that isolated Golgi membranes, ARF, coatomer proteins, GTP and fatty acyl-coenzyme A were necessary for the formation of COPI-coated vesicles. The myristolyated ARF protein first bound to the membrane, the coatomer then bound to activated ARF and led to membrane curving. Pinching off of vesicles needed acyl-coenzyme A (see Rothman *et al.* 1996). Spang and coworkers (1998) were able to produce from synthetic acidic phospholipids COPI-coated vesicles with Arf1p, a nonhydrolyzable GTP-analogue and the yeast coatomer without any additional factors. In mammalian cells the tails of p24-family proteins were additionally needed for Golgi-like liposomes to produce vesicles *in vitro* (Bremser *et al.* 1999).

1.5.7 GTP exchange factor for ARF proteins in the early secretion pathway

The cytosolic, soluble ARF-GDP is first recruited to membranes, and changed by GEF to membrane-bound form. All GEFs of ARFs contain a Sec7-domain. The yeast Sec7 protein operates in exocytic membrane traffic. In EM studies a mutant defective in Sec7 function had a block in normal Golgi progression resulting in tubularization of cargo-containing ER membranes (Deitz *et al.* 2000). Deitz and coworkers suggested these structures to be VTCs that failed to progress into Golgi structures without functional Sec7p. Furthermore, a Sec7-domain peptide added to an *in vitro* budding assay inhibited vesicle formation. Based on glycosylation defects, Franzusoff and Schekman (1989) showed Sec7p to be required for ER to Golgi transport. In indirect immunofluorescence microscopy, it associated to late Golgi membranes (Franzusoff *et al.* 1991). In *in vitro* assays, antibodies raised against Sec7p did not inhibit inclusion of soluble cargo into secretion vesicles, but inhibited the fusion of vesicles with the Golgi (Franzusoff *et al.*1992). Morin-Ganet *et al.* (2000) saw, in a Golgi morphogenetic study in the temperature sensitive *sec23-1* cell line after releasing a ten minute restrictive temperature block, as a first sign of Golgi formation clusters of small vesicles bridged with membrane tubules maturating to nodular clusters. This compartment, as well as tubular clusters of small vesicles were in immuno-EM decorated by Sec7p antibody.

Sec7p (230 kD; Achstetter *et al.* 1988) belongs to a family of otherwise unrelated large multidomain proteins, that contain the conserved 200 amino acid Sec7-domain. This domain, consisting of ten α-helices arranged in a cylinder, functions *in vitro* as a GEF for ARF proteins. In yeast, Sec7p, Syt1p, Gea1p and Gea2p contain the Sec7 domain (see Jackson and Casanova 2000). All these GEF proteins are sensitive to the fungal metabolite brefeldin A (BFA) (Peyroche *et al.* 1999, Jones *et al.* 1999). BFA prevented the GEF function by arresting the dissociation of GDP-ARF from the Sec7 domain. Prevention of ARF activation led to dissociation of the COPI coatomer from the membrane. In mammalian cells, BFA treatment induces tubularization of the Golgi along microtubules to ER exit sites. Both *cis-medial*-Golgi transmembrane proteins as well as matrix proteins are directed to ER exit sites after BFA treatment (Lippincott-Schwartz *et al.* 1990, Mardones *et al.* 2006). BFA also blocked anterograde traffic from the ER (Donaldson *et al.* 1992). In yeast the first observations of the effects of BFA were made using an ergosterol-deficient yeast strain (*erg6*), because wild type cells were inefficient in the uptake of the drug. BFA arrested growth (Vogel *et al.* 1993). CPY remained in the core glycosylated ER form and the α-factor in the ER form and a partially α1,6-mannosylated *cis*-Golgi form in the presence of BFA. Thus, BFA seemed to influence ER exit and Golgi transport in yeast.

Two other potential GEFs in the early secretory pathway, Gea1p and Gea2p, are functionally redundant, and recycle between the membrane and the cytosol. Deletion of both genes together was lethal (Peyroche *et al.* 1996). In the absence of the *GEA2* gene, inactivation of the Gea1 protein resulted in dispersion of the Golgi. Golgi markers concentrated to one large doughnut shaped structure and partially

to the ER (Peyroche *et al.* 2001). Gea2p is a multicopy suppressor of the coatomer *sec21-3* mutant and deletion of both *ARF1* and *GEA2* is lethal (Spang *et al.* 2001). *In vitro* studies showed that both Gea1p and Gea2p could support transport from the *cis*-Golgi to the ER, while Sec7p could not. These results suggest that Gea proteins are responsible for at least the nucleation of the retrotransport coat COPI on the *cis*-Golgi membrane. Temperature-sensitive mutations 200 bp upstream of the Sec7-domain of *GEA2* revealed a new function for the Gea2 protein. There was a clear defect in ER exit. Also in *in vivo* experiments *trans*-Golgi-localized GFP-fusion protein staining was lost, while the ER accumulated and early Golgi fusion protein marker staining was partially ER localized. Anterograde transport defects could be due to a retrotransport defect, but Park *et al.* (2005) could not find an obvious retrotransport defect in the *gea2* mutants, studied with several methods. This suggests that Gea2p has a direct role in ER exit and fusion of ER derived vesicles with the Golgi (Park *et al.* 2005).

A mammalian BFA-sensitive GEF, GBF1, homologous to Gea proteins, was studied using confocal imaging and photobleaching taking advantage of a fluorescent fusion protein. The marker seemed to cycle efficiently between the cytosol and membranes, since after photobleaching the marker appeared to be instantly recruited to the membranes. BFA treatment seemed to mobilize the marker to membranes (Lippincott-Schwartz and Liu 2006).

How these large GEF proteins are bound to membranes is not known, as no lipid-binding domain has been identified. One candidate for the recruitment of Gea proteins might be the *cis*-Golgi located integral membrane protein Gmh1p that interacts with a conserved domain of Gea proteins (Chantalat *et al.* 2003). Another candidate could be Sec24p that *in vitro* interacts with the C-terminal half of Sec7p (Deitz *et al.* 2000). Clearly, the localization of GEFs to sites where the ARF protein is activated and used for coatomer recruitment is an important spatial trigger of COPI coat polymerization.

1.5.8 ARF proteins in vesicle formation

In the GDP-bound form the myristoyl tail of ARF is more or less buried and the protein is bound with weak hydrophobic and electrostatic interactions to anionic lipids of the membranes. Exchange of GDP to GTP changes the conformation and liberates the myristoyl tail for interaction with membranes. Deletion of the N-terminal myristoyl binding site did not abolish GTP dependent binding of ARF to membranes, indicating that an additional membrane binding mechanism exists (Franco *et al*. 1996). Antonny *et al.* (1997) showed that the N-terminal 13 amino acids form an amphipathic helix, which is responsible for tight interaction with membrane phospholipids. The recruitment of ARF to membranes was reported to be dependent on the cytoplasmic tail of p23, a p24 family member. Gommel *et al.* (2001) photo-crosslinked ARF-GDP to the C-terminal tail of p23 and this interaction was competed by a dimeric soluble form but not by a momeric form of the same non-photolabile peptide. A multimeric form of p23 seemed to be the receptor for binding of inactive ARF to membranes. Upon activation of ARF by GTP exchange the interaction with p23 was lost (Gommel *et al.* 2001). Similar data for yeast interactions is not available. In yeast, deletion of all eight p24 genes is not lethal, but deletion of both p23 alleles from mouse led to embryonic lethality (Springer *et al.* 2000, Denzel *et al*. 2000).

GTP-bound activated ARF had affinity towards β-COP and γ-COP, as studied with photolabile phenylalanine replacements in different positions of the effector loop. Two-hybrid data suggested interactions between ARF-GTP and β- and ε-COP (Zhao *et al.* 1999, Eugster *et al.* 2000). Although ARF-binding was essential for coat polymerization, interaction between ARF and the coatomer was weak (Bremser *et al.* 1999). Additional interactions between the coatomer and the recycling cargo stabilized the coatomer on the membrane (Sohn *et al.* 1996). When the low GTPase activity of ARF was stimulated by its activator ARF-GAP, ARF-GDP was recruited to the cytoplasm. Studying fluorescent ARF fusion proteins and the coatomer in living

cells, Presley and coworkers (2002) saw different residence times on the membrane, as the coatomer was more persistent. This result suggests that the staying of the coatomer on the membrane relies also on other interactions after GTP hydrolysis. The GTP cycle was proven to be essential for cargo inclusion into retrotransport vesicles, since vesicles produced in the presence of a nonhydrolyzable GTP analog contained significantly less cargo (Nickel *et al.* 1998, Malsam *et al.* 1999, Pepperkok *et al.* 2000). Golgi enzymes like α1,2-mannosidase II were shown to be ten-fold concentrated to newly formed vesicles as compared with Golgi (Lanoix *et al.* 1999). ARF activation seemed to play a role also in inclusion of SNAREs into vesicles. The binding towards ARF-GTP was specific to the Golgi-ER SNAREs (Lee *et al.* 2005). Finally, inactivation of ARF leading to coat depolymerization after vesicle fission is necessary for the fusion of the vesicular membrane with the target membrane (Cukierman *et al.* 1995, Reinhard *et al.* 2003).

1.5.9 GAP proteins in COPI vesicles

The activity of GAP is clearly needed for the de-coating of vesicles as well as for cargo sorting and concentration into vesicles. How are vesicles formed if de-coating is occuring all the time? Is cargo stabilizing coat interaction to the membrane and forming vesicles when ARF dissociates from the membrane? In *in vitro* studies the COPI coat was formed without GAP protein, differing from COPII vesicles where the GAP protein Sec23p is part of the structure. Yeast has six putative GAP proteins, and double deletion of *GLO3* and *GCS1* is lethal. *GLO3* deletion was also lethal in combination with the *sec21-3* mutation and affected retrotransport from Golgi to ER (Poon *et al.* 1999). Glo3p interacted in a two-hybrid screen with Sec21p (γ-COP) and Sec27p (β'-COP), suggesting a role in retrotransport vesicle formation (Eugster *et al.* 2000). In Sec21p, the appendage platform seemed to be responsible for Glo3p binding (Watson *et al.* 2004).

In *in vitro* studies Yang and coworkers (2002) used full length Arf1p, GTP and the coatomer with isolated mammalian Golgi membranes and collected those into a pellet by centrifugation. Adding ARF GAP to resuspended membrane solution liberated vesicles that floated in a second centrifugation. Coatomer and GAP were found in floating fractions and pellet while Arf1p was found in the pellet. Earlier Ostermann and coworkers (1993) reported the equimolar composition of ARF and the coatomer on isolated vesicles produced with γ-GTP. With the γ-GTP Yang and coworkers could also detect ARF1 staining in floating vesicles, but the signature from ARF GAP was significantly reduced. Furthermore, they showed more GAP compared with the coatomer (3:1) in vesicles formed in the presence of GTP, suggesting a more structural and possibly initiating role for GAP in COPI vesicle formation. In yeast cells, also *in vivo* the interaction of Glo3p with the coatomer on vesicles was proven (Lewis *et al.* 2004).

Before insertion into forming COPI vesicles, GAP protein interacts with *trans*membrane cargo molecules like the KDEL receptor. Upon pH dependent substrate binding, KDEL receptors oligomerize leading to phosphorylation by PKA and GAP binding via its non-catalytic domain (Aoe *et al.* 1997, 1999, Cabrera *et al.* 2003). GAP was shown to bind directly also to the cytosolic tails of p24 proteins. The p24 proteins were thereafter sorted into a different pool of COPI-coated vesicles than mannosidase II and Golgi SNARE GS28, and these tails seemed to inhibit GAP activity (Lanoix *et al.* 2001). On the other hand, GAP activity is reported to be increased when the size of the vesicle reaches the correct diameter (60 nm). The GAP curvature-sensing motif doubles the rate of GTP hydrolysis (Bigay *et al.* 2003). Bigay and coworkers (2005) localized this conserved motif (40 amino acids) into the middle part of Glo3p and Gcs1p. The motif was unstructured in solution but formed an amphipathic helix on curved membranes, inserting itself between loosely packed lipids.

Lee *et al.* (2005), using Wbp1 proteins' KKXX-binding sequence known to recruit the WD40-domain of α-COP (Schröder-

Köhne *et al.* 1998), studied the sequential binding of the coatomer and GAP. They found that in solution GAP and the coatomer did not interact. Binding of GAP first to the tail of Wbp1 increased coatomer binding to the complex. Preincubation with KKXX peptides inhibited coatomer binding directly to the Wbp1-tail, but not to GAP-interacting with Wbp1 tail. GAP harbors two binding domains towards the COPI coat, the catalytic and non-catalytic domain. ARF-GTP binds to v-SNAREs, but not to other cargo, so that ARF-GAP non-catalytic site interaction to the coatomer in the presence of the ER retrieval cargo can have an initiating role in COPI vesicle formation.

1.5.10 Coatomer in retrotransport cargo harvesting

Eugster *et al.* (2004) showed that also the KXKXX-signature in Emp47 was bound to the coatomer. Another motif, WXXWY/F, joined to the cytoplasmic tail of a reporter protein ensured its ER localization via δ-COP interaction. This motif is used for ER retrieval of Sec71p (Cosson *et al.* 1998). Isolated COPI vesicles contained p24 family members as the main group of transmembrane cargo proteins (Stamnes *et al.* 1995). Some of these contain dibasic retrieval signatures, like KXKXX in Erv25p. The Emp24 protein contains, like its mammalian homolog p24, a di-phenylalanine (FF) motif known to bind COPII, but no KKXX signature. Mammalian p24-members lacking the KKXX motif were shown to bind β-, γ- and ζ-COP complex probably through the di-phenylalanine motif (Fiedler *et al.* 1996). Bethune and coworkers (2006) studied the p24 proteins' cytoplasmic tails that harbor FFXXBBXX, where B represents a basic amino acid, and found it to bind to two different sites in γ-COP, the N-terminal trunk domain and the C-terminal appendage domain. Dimerization of the tail was required for binding to γ-COP, while monomeric ER-resident proteins were bound through the KKXX motif to WD40 domains of the coatomer.

In vitro studies of mammalian p23 tetramer tails by Reinhard *et al.* (1999) revealed a conformational change induced by tail binding, leading to a more protease resistant form of γ-COP. The conformational change induced by binding of the oligomerized receptor to γ-COP is considered to lead to membrane deformation and finally to vesicle formation.

1.5.11 COPI vesicle formation *in vivo*

What is known? As discussed above all coatomer components are essential for growth. They assemble in the cytosol to a complex, and are then recruited onto

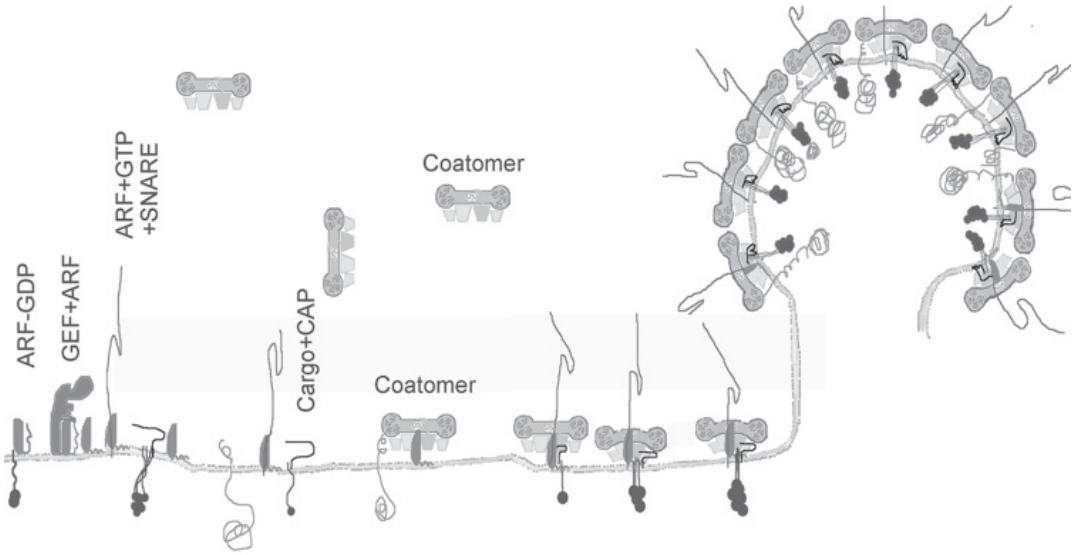

Figure 11. A schematic picture of events leading to COPI coated vesicle formation.

membranes, where they start to form a lattice upon ARF priming (Fig. 11). ARF proteins are recruited on the membrane maybe through weak interactions with putative cargo receptor tails, and form a tight interaction with the membrane after encountering a GEF-protein, at same time releasing the weak interaction from the p23 tail. Activated ARF binds to v-SNAREs and is ready to bind the coatomer. Initially, GAP is transferred to the membrane through its affinity to cargo tails, and this probably mediates the interaction of the coatomer γ subunit or WD40 domains to cargo when these complexes meet. Some cargo tails are receptor proteins and mediate GAP binding only in a multimeric phosphorylated form. ER resident membrane proteins that are monomeric bind through WD40 domains. GAP binds to the coatomer through interacting domains. It is probable that GAP can also directly associate with the coatomer on the membrane without initial cargo binding. Based on FRET assays, GAP and the coatomer cycle constantly on and off the membrane also under conditions where vesicle formation is blocked (Lippincott-Schwarz and Liu 2006). It seems that if correct cargo interactions are not available, ARF GTPase activity is induced by GAP more rapidly. And *vice versa*, GAP induction may be reduced by binding of GAP to p24 family tails. This gives time to form correct interaction with cargo tails, leading to a conformational change in the coatomer.

Insertion of ARF amphipathic helix in between lipid head groups increases outer membrane volume, leading to short tubule formation. At the tip of the membrane, curvature is sensed by the amphipathic helix of ARF GAP, which catalyzes with its zinc finger the ARF GTPase activity induction. GTP hydrolysis leads to release of ARF-GDP from the membrane and coatomer contact. The coatomer and GAP itself stay connected on the vesicle probably by interactions to cargo tails until the vesicle is formed. GEF and GAP activities fuel new ARF cycles at the forming neck of the vesicle to recruit more cargo and reduce cargoless coatomer assembly.

How the fission of the coated vesicle happens is not known. *In vitro* studies suggested that the yeast coatomer and ARF proteins were the only necessary factors. Clearly *in vivo* accessory factors are needed, such as palmitylation of proteins or lipids by acyl-coenzymeA. Liberated vesicles in mammalian cells are transported along microtubule and microfilament tracks by myosin motors to the ER (Valderrama *et al.* 2001 Duran *et al.* 2003), shedding the coat at some point before membrane fusion.

2 AIMS OF THE STUDY

Different temperature sensitive secretion mutants isolated by Novick and coworkers (1980) and in studies thereafter have led to a concept of essential core factors in the secretion route which starts to be a dogma. We tried to probe the variability in the early secretion pathway by using non-functional temperature-sensitive forms of essential proteins as well as to map new determinants for active ER exit by studying endogenous glycoprotein secretion.

3 MATERIALS AND METHODS

Experimental methods used in this thesis work are listed in table 1 and are described in detail in the original publications. *S. cerevisiae* strains with their relevant genotypes are listed in table 2 and schematic picture of the reporter proteins is presented in Fig. 12.

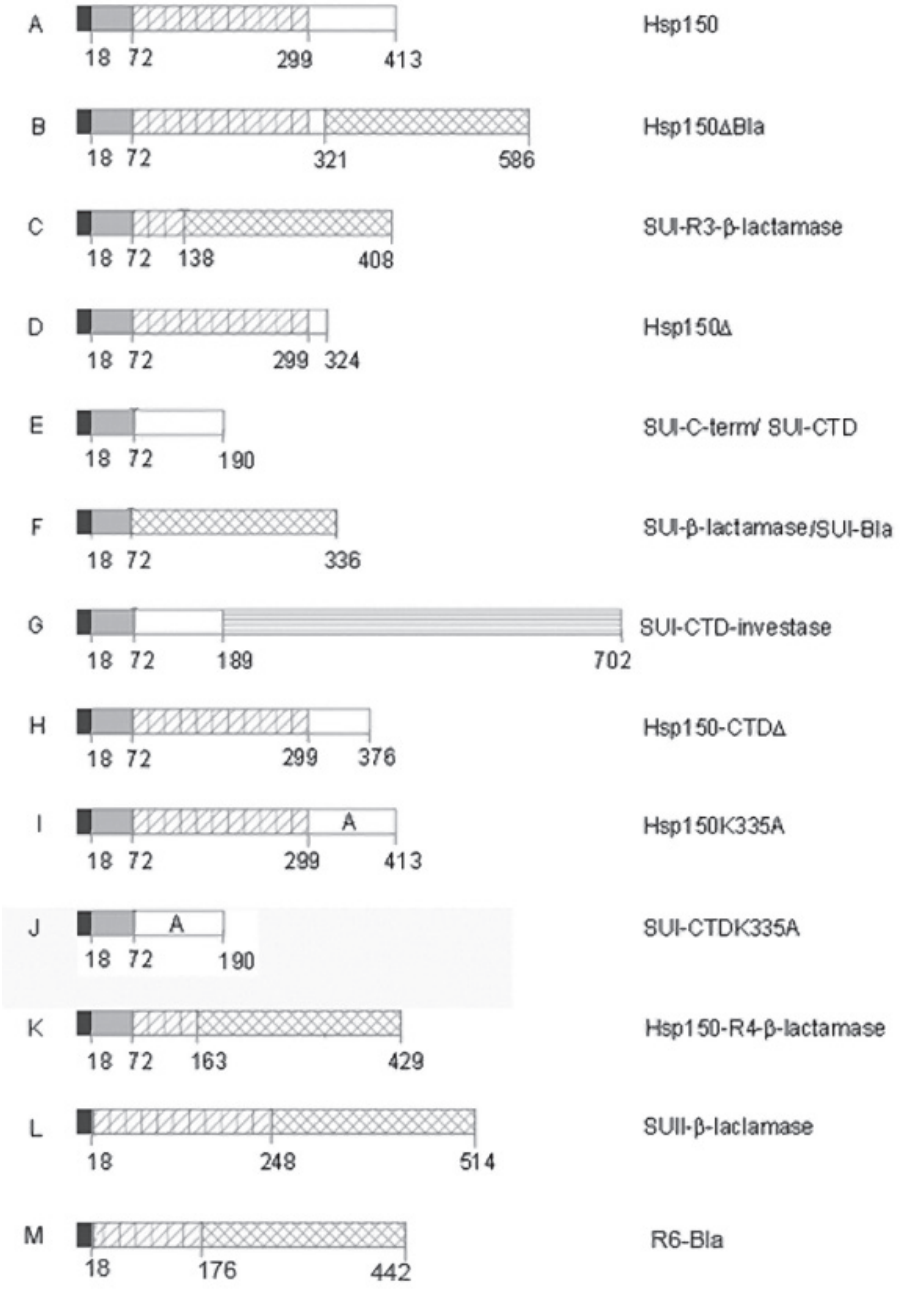

Figure 12. A schematic picture of Hsp150 constructs. Hsp150 consists a signal sequence aa 1-18 (black), subunit I aa 19-72 (SUI; grey), and subunit II aa 73-413 (SUII). SUII is divided into repetitive region (diagonally striped boxes) and C-terminal domain (white). β-Lactamase fusion part to Hsp150 is cross hatched. Invertase protein fusion part is horizontally striped.

Table1. Methods used in the study.

ATPase assay	II
β-lactamase assay	I, III
Centricon concentration	II, III
Dialysis	II
Immunofluorescence microscopy	I, III
Immunoprecipitation	I, III
Intein fusion protein purification	II
Invertase assay	I, III
Mass spectrometry	III
Metabolic labeling with [^{35}S]-methionine-cysteine	I, II, III
Nucleotide sequencing	I, II, III
Ni-NTA- agarose column protein purification	III
Plasmid construction	I, II, III
Reversed phase HPLC	III
SDS-PAGE	I, II, III
Site directed mutagenesis	II
Western analysis	II
Yeast gene disruption	I
Yeast mating and tetrad dissection	I, II
Yeast transformation	I, II, III

Table 2. Yeast strains used in the study.

Strain code	Relevant genotype	Source
H1		R. Schekman
H3	*sec7-1*	R. Schekman
H4	*sec18-1*	R. Schekman
H10	*sec7-1*	Schekman
H22	*leu2-3,112 kar2-159*	M. Rose
H23	*hsp150*::*URA3*	Russo *et al*. 1992
H211	*sec2-56*	YGSC
H229	*sec12-4*	Novick *et al*., 1980
H230	*sec13-1*	Novick *et al*., 1980
H235	*sec21-1*	Novick *et al*. 1980
H238	*sec23-1*	Novick *et al*.1980
H245	*ura3-1 leu2-3,112 trp1-1*	K. Kuchler/J. Thorner
H247	*ura3-1 leu2-3,112 trp1-1*	K. Kuchler/J. Thorner
H259	*sec63-1*	R. Schekman
H264	*ypt1*	D. Gallwitz
H335	*ura3-52::[URA3, HSP150Δ-BLA]*	Simonen *et al.*1994
H340	*sec7-1 ura3-52::[URA3, HSP150Δ-BLA]*	Simonen *et al.* 1994
H393	*sec18-1 ura3-52::[URA3, HSP150Δ-BLA]*	Simonen *et al*.1994
H430	*hsp150*::*URA3 leu2::[LEU2, HSP150Δ]*	I
H440	*sec18-1 hsp150*::*URA3 leu2::[LEU2, HSP150Δ]*	I
H481	*sec23-1*	R. Schekman
H484	*sec23-1 ura3::[URA3, HSP150Δ-BLA]*	III
H675	*leu2::[LEU2, HSP150Δ-BLA]*	I, III
H677	*sec18-1 leu2::[LEU2, HSP150Δ-BLA]*	III
H680	*kar2-159 leu2::[LEU2, HSP150Δ-BLA]*	III
H786	*his3 sec21:: HIS3 leu2 [LEU2, CEN6 sec21-3] ura3::[URA3, HSP150Δ-BLA]*	III
H791	*ura3::[URA3, HSP150-R4-BLA]*	III
H792	*his3 sec21:: HIS3 leu2 [LEU2, CEN6 sec21-3] ura3::[URA3, HSP150-R4-BLA]*	III
H827	*sec18-1 ura3::[URA3, HSP150-R4-BLA]*	III
H830	*sec21-1 leu2*	Hosobuchi *et al*., 1992
H831	*sec21-1 leu2::[LEU2, HSP150Δ-BLA]*	III
H834	*sec21-1 leu2::[LEU2, HSP150Δ-NGFRe]*	III
H839	*leu2::[LEU2, SUI-BLA]*	I, III
H840	*sec18-1 leu2::[LEU2, SUI-BLA]*	I, III
H842	*sec21-1leu2::[LEU2, SUI-BLA]*	III
H849	*ura3-1 hsp150*::*URA3 trp1-1::[TRP1, HSP150-His6]*	III
H890	*leu2::[LEU2::SUII-BLA]*	III

Table 2 continuing

H911	*his3-11,15 pep4*::*HIS3* prb1-*Δ leu2-3,112*::*[LEU2, SUI-BLA]*	III
H940	*ura3-52 trp1- 901 leu2-3, URA3*::*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ*	Clonetech
H973	*sec21-1 leu2*::*[LEU2, SUII-BLA*]	III
H975	*leu2-3,112 erg6Δ::LEU2*	D. Gaber
H1064	*sec13-1 leu2*::*loxP-KanMX-loxP*	I
H1065	*sec13-1 leu2*::*loxP-KanMX-loxP [LEU2, HSP150Δ-BLA]*	I
H1067	*sec13-1 leu2*::*loxP-KanMX-loxP [LEU2, SUI-BLA]*	I
H1102	*sec31-2*	C. Kaiser
H1107	*sec13-1 leu2*::*loxP-KanMX-loxP [LEU2, HSP150 Δ]*	I
H1233	*hsp150*::*loxP-KanMX-loxP*	I
H1234	*hsp150*::*loxP-KanMX-loxP*	I
H1236	*sec13-1 trp1*::*loxP-KanMX-loxP*	I
H1284	*sec13-1 ura3*::*loxP-KanMX-loxP*	I
H1295	*ypt6 leu2-3, 112*	J. Warner
H1359	*sec21-1 R6-Bla*	
H1400	*sec13-1 ura3*::*loxP-KanMX-loxP [URA3, SUI-R3-BLA]*	I
H1429	*sec13-1 trp1*::*loxP-KanMX-loxP [TRP1, SUI-CTD]*	I
H1431	*sec63-1 ura3-52 [URA3, SUI-R3-BLA]*	I
H1432	*sec18-1 ura3-52 [URA3, SUI-R3-BLA]*	I
H1433	*sec7-1 ura3-52 [URA3, SUI-R3-BLA]*	I
H1508	*hsp150*::*loxP-KanMX-loxP trp1-1[TRP1, SUI-CTD]*	I
H1540	*hsp150*::*loxP-KanMX-loxP trp1-1*::*[TRP1, SUI-CTD-invertase]*	I
H1541	*sec13-1 trp1*::*loxP-KanMX-loxP [TRP1,SUI-CTD–invertase]*	I
H1542	*sec18-1 trp1-289*::*[TRP1, SUI-CTD–invertase]*	I
H1545	*sec13-1 leu2-3, 112 [LEU2, HSP150Δ] hsp150*::*loxP-KanMX-loxP*	I
H1639	*sec24-1ura3 leu2-3,112 trp-1*	II
H1642	*sec13-1*	II
H1780	ura3-52 *emp47 ::loxP-KanMX-loxP*	euroscarf
H1801	*emp47 [URA3, HSP150Δ-BLA]*	
H1872	*trp1-1 ura3-1 hsp150::URA3 TRP1::HSP150CTDΔ*	II
H1890	*sec21-1 ypt6 [LEU2, HSP150Δ-BLA]*	
H1920	*ypt6[LEU2, HSP150Δ-BLA]*	
H1924	*sec24-1 trp1-1 hsp150::URA3*	II
H1925	*sec24-1 trp1-1 hsp150::URA3 TRP1::HSP150CTDΔ*	II
H1963	*ura3-52 trp1- 901 leu2-3, URA3*::*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ* [pKTH5199*; TRP1*]	
H1964	*ura3-52 trp1- 901 leu2-3, URA3*::*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ* [pKTH5200*; TRP1*]	
H1965	*ura3-52 trp1- 901 leu2-3, URA3*::*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ* [pKTH5201*; TRP1*]	
H1971	*ura3-52 trp1- 901 leu2-3, URA3*::*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ* [pKTH5207*; TRP1*]	
H1975	*leu2-3,112 trp1-1* [pKTH5208*, LEU2]*	
H1976	*leu2-3,112 trp1-1* [pKTH5209*, LEU2]*	
H1977	*leu2-3,112 trp1-1* [pKTH5210*, LEU2]*	
H1978	diploid strain H1963xH1975	
H1979	diploid strain H1963xH1976	
H1980	diploid strain H1963xH1977	
H1981	diploid strain H1964xH1975	
H1982	diploid strain H1964xH1976	
H1983	diploid strain H1964xH1977	
H1984	diploid strain H1965xH1975	
H1985	diploid strain H1965xH1976	
H1986	diploid strain H1965xH1977	
H2057	*sec23-1 trp1-1 ura3-52 hsp150::URA3 TRP1::HSP150CTDΔ*	II
H2126	*sec24-1 trp1-1 hsp150::URA3 TRP1::HSP150Q400A*	II
H2127	*leu2-3,112 trp1-1 [pKTH5225, LEU2]*	
H2128	diploid strain H1964XH2127	
H2264	*ura3-1 trp1-1 hsp150::URA3 TRP1::HSP150K355A*	II
H2265	*sec24-1 ura3-1 trp1-1 hsp150::URA3 TRP1::HSP-150K355A*	II

Additional plasmid constructs and yeast strains

A PCR fragment for constructing a fusion protein to the Gal4 promoter binding domain for N-terminal fragment of Sec24p (amino acids 1-180) was amplified with oligos (Sigma) 5'-AATTACATATGTCTCATCACAAGAAACGT-3' and AAATTGTCGACTTAATCTGGAGACGCATTGGACAA using pKTH5178 (Karhinen *et al*. 2005). PCR fragments were digested with *Sal*I and *Nde*I and ligated with similarly digested pAS1 (Clonetech, Palo Alto, USA) to produce pKTH5199. The middle domains of Sec24p (amino acids 181-637) were amplified from pKTH5178 with oligos 5'-AAATTCATATGTATATCAGGTCGACCTTGAAC-3' and 5'-AAATTGGATCCTTAGTCAGCAGATGCGTAGACTTC-3' in a PCR reaction and after *Sal*I and *Bam*HI digestion ligated to *Sal*I and *Bam*HI-digested pAS1 to produce pKTH5200. pKTH5201, containing the Gal4 DNA binding domain fusion to the C-terminal fragment of Sec24p (amino acids 638-926) was made by ligating a *Nco*I and *Sal*I-digested PCR fragment, made with oligos 5'-AATTACCATGGGACAATTAGCTATAGCGTCGTTT-3' and 5'-TTAAAGTCGACTTATTTGCTAATTCTGGCTTT-3' from pKTH5178 and pAS1 together. Oligos 5'-AATTAGCCATGGAAAATCATCTGTATCTCATCAC-3' and 5'-AATAACTCGAGTTAATTATTAAGC-3' were used to amplify a DNA fragment encoding amino acids 1709-1771 of the Sec7 protein from H1 genomic DNA isolated with the Wizard® Genomic DNA Isolation Kit (Promega, Madison, USA). Amplified PCR fragments as well as the cloning vector pACT-2 (Clonetech, Palo Alto, USA) were digested with *Nco*I and *Xho*I and ligated together to create pKTH5209. Similarly, oligos 5'-AATAACCATGGGAGGCAATTCCGTTCCGACGGTA-3' and 5'- AATAACTCGAGTTAATCAGTAGAAAGGTATAA-3' were used to amplify the amino acids 1772-2009 of the Sec7p. Amplified PCR fragments were digested with *Xho*I and partially with *Nco*I and ligated to cloning vector pACT-2 and named pKTH5210. pKTH5210 was digested from internal *Nco*I site to create a shorter fusion protein (amino acids 1941-2009 of Sec7p) named pKTH5208. Mutated form of the Sec7p C-terminus was made with two overlapping PCR fragments done with oligos 5'-TAGTTGATAAAATACCAAACGGGGGGAAACAAGAAACGAGTGCTG-3' and 5'- AATAACTCGAGTTAATCAGTAGAAAGGTATAA-3' and 5'-AATAACCATGGGAGGCAATTCCGTTCCGACGGTA-3' and 5'-CAGCACTCGTTTCTTGTTTCCCCCCGTTTGGTATTTTATCAACT-3'. PCR fragments were mixed, heat-separated and annealed together and single stranded DNA strands were filled with DNA-polymerase I (Promega, Madison, USA). Double stranded annealed DNA-fragments were digested with *Eco*RI and *Xho*I and ligated to *Eco*RI and *Xho*I-digested pKTH5210 to create pKTH5225. Plasmid pHTH5207 was created by joining together *Nco*I-digested pAS1 and PCR fragment made with oligos 5'-TTATTCCATGGGATCCGCTTCTGCAGCCGCTACC-3' and 5'-AATTCTCGAGTTAACAGTCTATCAAATCGAT-3', blunted with DNA polymerase and ligated.

Plasmids pKTH5199, pKTH5200, pKTH5201 and pKTH5207 were transformed into a yeast strain H940 to create H1963, H1964, H1965 and H1971, respectively (Table 2). Similarly pKTH5209, pKTH5210, pKTH5208 and pKTH5225 were transformed into yeast strain H247 to create H1975, H1976, H1977 and H2127, respectively (Table 2). Diploid strains were done by mating H1963 with H1975, H1976 and H1977 creating H1978, H1979 and H1980, respectively (Table 2). Mating H1964 with H1975, H1976 and H1977 created H1981, 1982 and H1983, respectively (Table 2). Mating H1965 with H1975, H1976 and H1977 created H1984, H1985 and H1986, respectively (Table 2). Finally, H2128 was done by mating H2127 with H1964 (Table 2).

R6-bla cloning was done by joining together a *Kpn*I and *Eco*RI-digested PCR fragment made with oligos 5'-ATATAGGTACCGAGCTCTTAGCCCCAAAGAGCACC-3' and 5'-AATGAATTCGGAAGTAGCAGTAGTAGCTTG-3' using pKTH4705 as a template and a *Kpn*I and *Eco*RI-digested vector, pKTH4705. After *Bam*HI digestion a fragment containing under HSP150 promoter the HSP150 signal sequence and six last PIR repeats joined to beta-lactamase was ligated to *Bam*HI-digested integrative vector pFL26, creating pKTH4981. PKTH4981 was transformed into yeast strain H830 creating H1359 (Table 2).

Yeast strain H1890 was obtained after mating of yeast strains H831 together with H1295, sporulation and selection of double temperature sensitive mutant with beta-lactamase expression (Table 2). H1920 was obtained by transforming pKTH4545 (Simonen *et al.* 1994) into H1295 (Table 2).

Two-hybrid screening

H1971 was transformed with electroporation (www.bio.com/protocolstools/ protocol.jhtml) with FRYL-genomic library (Fromont-Racine *et al.* 1997) to obtain 5 x 10^6 colony forming units on selection plate (SC-his,-leu,-ura,-trp) supplemented with 3,3 g/l of 3-amino-1,2,4-triazole (Sigma-Aldrich). Colonies appearing on selection plate were tested for capability to grow on selection and for beta-galactosidase activation. LacZ assays were performed following the Clonetech Matchmaker manual. Plasmids were isolated and preys were identified by sequencing. Retransformations to yeast strain H940 was confirmed by Western blotting.

Other methods

Metabolic labeling with 20 µCi/ml [^{35}S]-methionine/cysteine (1000 Ci/mmol; Amersham International, Buckinghamshire, UK) and immunoprecipitation with antisera against Hsp150 (1:400), CPY (1:100) and beta-lactamase (1:100) were done as described previously Paunola *et al*. (1998). In Western blotting, monoclonal antibody against Gal4-activator domain (Clonetech, Palo Alto, USA) (1:2500 dilution) and Bgl2p-antiserum (1:20000) were used, secondary antibodies were hoseradish peroxidase (HRP)-conjugated (1:10000 dilution Promega, Madison, USA) and the detection was with an ECL kit (Amersham International, Buckinghamshire, UK). Phosphoimager quantitation was carried out using tina 2.0 software. BFA was diluted into ethanol to obtain a 10 mg/ml stock solution and used at 20 µl/ml.

4 RESULTS AND DISCUSSION

4.1 Endogenous reporter proteins

In studies of temperature sensitive yeast mutants, cell samples are incubated at restrictive temperatures (37 ºC), touching the upper limits of the normal growth conditions of yeast, which leads to increased expression of heat shock proteins. One secretory protein in the yeast culture medium was clearly induced after heat treatment. Russo and coworkers (1992) isolated this protein from the culture medium, cloned its gene and named it as heat shock protein 150 (Hsp150), based on increased expression by heat and migration in SDS-PAGE analysis. It belonged to a small family of non-essential secreted cell wall located proteins harboring PIR-repeat(s) (Toh-e *et al.* 1993, Mrsa, *et al.* 1997, Mrsa and Tanner 1999). The N-terminal 18 amino acids of Hsp150 (Fig. 12A) acting as a signal sequence guided the polypeptide (413 amino acids) strictly post-translationally to the ER (Paunola *et al*. 1998), where extensive O-glycosylation of the polypeptide was initiated by addition of mannoses to serine or threonine residues located in subunit I (SUI) and subunit II (SUII) (Russo *et al*. 1992, Jämsä *et al*. 1994). The N-terminal part of SUII consisted mainly of 11 PIR repeats (usually TAAAVSQIGDGQVQATTKT) that had no obvious structure in circular dicroism (CD) spectroscopy as a glycosylated form (Jämsä *et al*. 1995). Between the six first repeats, other sequences than repeat amino acids (four short flanking areas) were also present. The C-terminus of the polypeptide harbored four cysteine residues and at least one sulfhydryl bridge between β sheets (Jämsä *et al*. 1994, 1995). During secretion the protein is most probably modified like many other O-glycosylated secretory proteins in the Golgi by extension of linear mannoses by α1,2 linkage (Mnt1p; Hausler *et al*. 1992, Lussier *et al*. 1996) and α1,3-linkage (Mnn1 family; Romero *et al*. 1999). The ratio of mannose extensions (di-, tri-, tetra-, penta-saccharides) after β-elimination of the mature protein (SUII) harvested from the culture medium was 4:1:1:1, indicating di-mannosylation to be the most common modification (Jämsä *et al.* 1995). This type of mannosylation retarded the approximately 34 kD apoprotein in SDS-PAGE analysis to run like a 150 kD protein. A second type of modification that happened to Hsp150 was a proteolytic cleavage between SUI and SUII at the Kex2p protease recognization site, leading to 342 and 53 amino acid long SUII and SUI. SUI was bound to the C-terminal domain of SUII in the native conformation of the protein isolated from the culture medium (Russo *et al*. 1992).

We examined Hsp150 glycan maturation in more detail from proteins harvested from different types of well-known secretion mutant strains, based on the migration of the different forms in SDS-PAGE (Fig. 13). Different forms of Hsp150 have already been documented in many mutants, but not in a single experiment. Cell samples in a fresh medium were adjusted to 37 ºC for 15 minutes to inactivate temperature sensitive forms of proteins, prior to 5 minute metabolic labeling with radioactive [^{35}S]-methionine/cysteine mixture. Pulse sample labeling was terminated and parallel samples were chased in the presence of cycloheximide (CHX) for 60 minutes to prevent further protein synthesis. Transports of proteins were terminated by the addition of sodium azide. Culture media and cell samples were separated by centrifugation and Hsp150 proteins were immunoprecipitated with Hsp150 antiserum. Hsp150 secretion to culture medium without secretion block took place with a half time of 2 minutes (Jämsä *et al.* 1994). In pulse samples (Fig. 13, lanes 1 and 2) from wild type cells (H1) we detected only the mature form (150 kD) and a portion of it was in the culture medium (lane 1). After a 60 minute chase, practically the entire signal came from the culture medium (lane 3).

In *sec18-1* strain (H4) at restrictive temperature, where all membrane fusion events are prevented due to unsolved *cis*-paired SNAREs after previous fusion (Novik *et al.* 1981, Mayer *et al.* 1996, Kosodo *et al.* 2003), Hsp150 was totally intracellular. The pulse-labeled form harbored mannoses added to several potential acceptor residues, and run in the gel as an 89 kD protein (Fig.

13 lane 5, Jämsä *et al.* 1994). As the chase continued for 60 minutes, Hsp150 became retarded in the SDS-PAGE, running as a 100 kD form. The apparent increase of the size is probably due to addition of more mannosyl residues to the polypeptide chain trapped in the ER lumen. In paper chromatography after β-elimination of labeled O-glycans from proteins harvested from the *sec18-1* strain grown under restrictive conditions, a di-mannose pool was also detected (10 %, Hasselbeck and Tanner 1983). Studying O-glycans of the chitinase protein from *sec18-1* strain Kuranda and Robbins (1991) showed that more than half of the extracted mannoses were di-mannoses, but longer chains were totally lacking. We interpreted the size difference of the marker protein Hsp150 to be due to extensions of some mono-mannoses to di-mannoses by newly synthesized Golgi enzymes trapped in the ER lumen in *sec18-1* mutant conditions, since secretory proteins retarded due to folding problems without secretion block are not further glycosylated during an extended stay in the ER lumen (for example: Fig. 16, an ER form of R6-Bla at permissive temperature). The *sec18-1* mutation was originally characterized as a vesicle accumulating mutant. Vesicles were counted after a one hour incubation at restrictive temperatures and the amount of vesicles increased during secretion block by approximately 6.5 fold (Kaiser and Schekman 1990). Still the main portion of the cargo seemed to reside in the ER lumen based on enzymatic staining of a HRP-fusion protein in EM studies (Suntio, Bastos and Makarow, manuscript, data not shown).

In an anterograde transport coat mutant (COPII, *sec23-1* strain, H481) preventing ER exit (Novik *et al.* 1981, Yoshihisa *et al.* 1993), Hsp150 was kept inside the cell. Its migration in SDS-PAGE was further retarded (110-130 kD) compared with the *sec18-1* chased form (Fig. 13 lane 7, Jämsä *et al.* 1994). The change of the protein's migration was gradual, increasing with the chase time (I, Fig. 3B). Some Golgi transferases have been shown to recycle through the ER (Todorow *et al.* 2000). The COPII coat mutation seemed not to influence the retrotransport of transferases. Karhinen and Makarow (2004) showed that some of the recycled transferases were active even as an ER trapped form. The change in the size of Hsp150 is a consequence of newly synthesized and recycled *cis* Golgi transferase activity that extended more and more of the protein-connected single mannoses to di-mannoses in the ER lumen. In the *sec7-1* strain (H10) (Novick 1981, Franzusoff and Schekman1989) Hsp150 was in an internal mature form (Fig. 13. lane 8) as in the control strain *sec2-56* (H211) (Fig. 13, lane 9), which is a late transport mutant that accumulated *post* Golgi vesicles (Walch-Solimena *et al.* 1997).

Another widely used marker protein is vacuolar CPY. CPY in normal conditions, after translocation to ER, obtains four N-glycans and runs in SDS-PAGE as a 67 kD form (p1, ER form). In Golgi, glycans

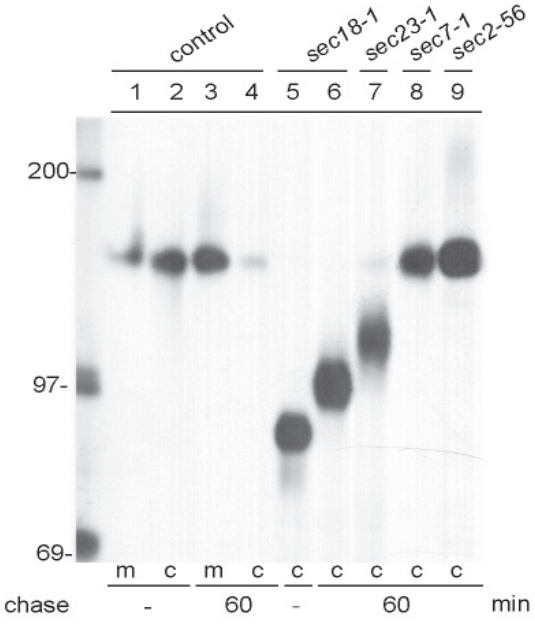

Figure 13. Endogenous Hsp150 secretion forms in different strains after 5 minute metabolic labeling and/ or 60 minute chase, resolved in SDS-PAGE after immunoprecipitation. c; cell lysate sample, m; culture medium sample. Protein size standards are indicated on the left.

are further extended. Och1p adds first α1,6-linked mannose in *cis*-Golgi (Dean *et al*. 1999), and thereafter more mannoses are added with α1,2- and α1,3-linkages to make the 69 kD Golgi form (p2). Arrival to the vacuole can be followed by the appearance of the mature 62 kD form after a proteolytic cleavage by the Pep4 protease (Stevens *et al*. 1982).

Different forms of CPY are shown as a control at permissive temperatures from another COPII mutant, *sec13-1* (H230), in similar pulse chase experimental settings as previously with different chase times. Cell lysates were immunoprecipitated with CPY antiserum. After a five minute metabolic labeling with radioactive [^{35}S]-methionine/cysteine only p1 form was seen (I, Fig. 2Ab, lane 1). In ten minute chased cell lysate different forms of CPY had appeared. The ER located p1 form was still the most prominent, but also the p2 Golgi form was seen and some signal of the vacuolar mature form as well (I, Fig. 2Ab, lane 2). After a 20 minute chase the main form was the mature form (I, Fig. 2Ab, lane 3) and in 30 and 40 minute chased samples only the mature form was seen (lanes 4 and 5).

These mobility differences of endogenous reporter proteins are used throughout this study to demonstrate the location of the reporters in different temperature sensitive anterograde and retrograde mutants.

4.2 COPII independent transport

4.2.1 Hsp150 secretion in non-functional COPII coat mutant strains

We studied Hsp150 behavior also in COPII coat temperature sensitive mutants *sec31-2* (H1102), *sec12-4* (H229) and *sec13-1* (H230). Cells were preincubated, labeled, and CHX chased for various time periods. Cell lysates and culture medium samples were immunoprecipitated with Hsp150 antiserum prior to SDS-PAGE analysis as previously. In the *sec31-2* strain after the pulse only a form of around 100 kD was present in the lysate, which during different chase times developed into 110-130 kD forms (I, Fig. 3A). Only a very weak signal could be detected from the culture medium sample after a 40 minute chase (lane 8). Thus, at restrictive temperatures in the COPII mutant *sec31-2* our marker protein was kept in the ER lumen. In the *sec12-4* strain, the results were the same; the ER exit of Hsp150 was inhibited as expected (data not shown).

In the temperature sensitive *sec13-1* strain labeled at restrictive temperatures as before, a significant portion (38% as estimated by PhosphorImager) of the Hsp150 signal matured already within the 5 minute pulse (I, Fig. 1A, lane 2). After a 15 minute chase the signal was detected also from the culture medium (lane 3), while the internal signal was reduced (lane 4). Also the size of the internal ER form slowly grew during the chase as in other COPII mutants, but the size of the mature form was uninfluenced. As the chase proceeded, more and more of the Hsp150 matured and was externalized to the culture medium. In Fig. 1B (I) the relative estimates of Hsp150 mature form after PhosphorImager quantification are shown at different chase times. At the end of the chase period (60 minutes) 82% of the signal was mature and most of it came from the culture medium samples. We repeated the pulse chase experiment extending the preincubation time at restrictive temperature to 60 minutes prior to labeling and got similar results (I, Fig. 1B). Although the secretion rate of Hsp150 was significantly reduced in the *sec13-1* strain (maturation half time around 7 minutes), secretion was still quite complete. More recently, Hsp150 was shown to be secreted to the culture medium with similar kinetics also from the non-functional COPII strain *sec24-1* (Fatal *et al.* 2004).

4.2.2 Other reporter proteins in the COPII mutant *sec13-1*

To verify that the *sec13-1* mutant was not just leaky, leading to partial secretion, we assayed CPY secretion in the pulse chase experiment from parallel samples of the same culture. The ER form of CPY (p1) persisted during the tested chase time, up to 40 minutes (I, Fig. 2Aa, lanes 1-5).

We also analyzed another marker protein, invertase, by using its enzymatic activity. The production of this sucrose

hydrolyzing non-essential soluble periplasmic enzyme form of invertase (SUC2) is induced by starvation and repressed by glucose, which made it convenient reporter for cell biological studies (Carlson and Botstein 1982). Internal activity accumulated in the *sec13-1* strain after the induction of invertase expression at the restrictive temperature (I, Fig. 2B). At the permissive temperature the internal activity remained on a basal level and external activity increased during the assay time. We concluded that the *sec13-1* allele at the restrictive temperatures was capable of blocking the transport of other marker proteins at the level of ER exit, based on the CPY ER form and previous results of Novick *et al.* (1980) reporting the invertase transport block at the level of ER exit. We also confirmed their results in an invertase activity gel (not shown) and by activity measurements separating internal and external activity. Those same cells secreted Hsp150 to the culture medium indicating that Hsp150 transport was not due to mutant leakiness.

4.2.3 Possibilities in the anterograde transport of the biosynthetic cargo in the absence of functional Sec13p

4.2.3.1 Essentiality of Sec13p

The *sec13-1* mutation is a substitution of serine (224) by lysine (Pryer *et al.* 1993). At restrictive temperatures, the mutant protein was seemingly unable to support vesicle formation from ER exit sites since CPY and invertase were retained in the ER. Sec13p function seemed to be crucial for the coat polymerization, based on structural studies (Stagg *et al.* 2006). Sec31p/13p formed the outer layer of the coat (Matsuoka *et al.* 2001). Since the role of Sec13p in the COPII coat formation seemed to be central, joining molecules together, its total absence appeared to be impossible in theory, but still feasible in practice. A viable deletion strain of *SEC13* was achieved by mutating an additional gene, either *BST1, BST2/EMP24* or *BST3* (Elrod-Erickson and Kaiser 1996). The authors suggested that *BST* gene products were negative regulators of incorrectly coated COPII vesicle formation. Belden and Barlowe (2001) have shown an interaction of Emp24p tails with the Sec31/13p complex *in vitro*. Emp24p is known to bind both coat complexes Sec23/24p and Sec13/13p. Possibly, Sec24p mediated the interaction of Emp24p to Sec13p and this interaction excluded other Sec13p homologs in normal COPII coat formation. The *BST3* gene has not been cloned, and Bst1p functioned at least in the deacylation of GPI anchors (Tanaka *et al*. 2004). Additionally, *BST1* deletion retarded the ER exit of a transmembrane protein (Haass *et al*. 2007), which might indicate a more universal task in protein ER exit.

4.2.3.2 Possible role of Sec13p homologs

Since Sec13p seemed to be so central to the vesicle coat structure formation, it seems unlikely that the coat could be built without joining Sec31 molecules together, not even in the *SEC13* deletion strain. The closest homolog to Sec13p is a non-essential seven-blade WD40 fold-containing Seh1p (50% similarity, Siniossoglou *et al.* 1996). Seh1p and Sec13p are found in a nuclear pore complex together with Nup84p, Nup85p and Nup120p. An interesting theory of endomembrane development based on protein fold similarities suggested that membrane-bending nuclear pore complexes and transport vesicle forming coats have developed from a common origin (Devos *et al.* 2004). Devos and coworkers (2004) suggested that the structures of the seven proteins forming a core building block of the nuclear pore, termed the Nup84 subcomplex in yeast, fold as α solenoids similar to Sec31p and as WD40 repeats similar to the outer coat of the COPII complex. Possibly, Seh1p could replace Sec13p function in the ER exit coat of Hsp150, and CPY or invertase is not included in these vesicles. Perhaps the vesicles where Hsp150 is transported at least in heat stress situations lack Bst proteins and therefore allow another WD40 homolog to replace the mutant Sec13p. Alternatively, Hsp150 transport vesicles could constantly use another WD40 protein.

Another homolog for Sec13p is Lst8p, a WD40 fold protein, needed for regulation

of amino acid uptake responding to nutrient availability and cellular stresses through the TOR-complex (Roberg *et al.* 1997, Chen *et al.* 2003 Cardenas *et al.* 1999). *Sec13-1* and *LST8*Δ are synthetically lethal (Roberg *et al.* 1997) leading to the possibility that these proteins functioned in the same process. Hsp150 is not an essential gene and all three of its closest homologs can be deleted without a great impact on vitality under laboratory conditions (Toh-e *et al.* 1993). The synthetic lethality between *SEC13* and *LST8* genes is probably not induced by the defect in Hsp150 transport, but is a consequence of malfunction of the amino acid uptake. The putative roles of the Sec13p homologs have not been investigated further.

Yet another possibility is that the mutant form of Sec13p is able to support Hsp150 insertion into vesicles, but not the packing of other cargo proteins. This theory however does not explain the viability of the SEC13 deletion strain, which strongly supports the existence of a functional homolog.

4.2.3.3 Transport without conventional vesicle formation?

In mammalian cells very large polymers are transported out of the ER, such as collagen that does not fit inside the widest coated transport vesicle. Mironov *et al.* (2003) studied by EM and tomography collagen and VSVG transport and reported that saccular carriers detaching near ER exit sites contained the cargo, without COPII coated vesicle formation. COPII components were essential for saccular carrier formation and cargo concentration, but no signal of coat formation on the carrier was detected. Perhaps functional Sec13p is not needed, if a real curved vesicle is not formed and Hsp150 exits from the ER in multi-shaped containers. There is not a solid proof of non-vesicular (other than 50nm vesicles) transport out of the yeast ER, except autophagosomes (Ishihara *et al.* 2001). Autophagosome formation from ER membranes is shown to need Sec23/24p function but to be independent of Sec31/13p function. Hsp150 was totally dependent on Sec31p. Thus, autophagosome formation is not the solution for the Sec13p independent transport of Hsp150.

4.3 The determinant in Hsp150 for ER exit without functional Sec13p

4.3.1 Transport of N-terminal repetitive portion is Sec13p dependent

To map the feature in Hsp150 leading to Sec13p independent transport, we assayed different parts of Hsp150 for the capability of transport. All Hsp150 constructs used in this study were under control of the Hsp150 promoter. First we used a well established fusion protein, Hsp150Δ-β-lactamase (Hsp150ΔBla; Fig. 12B; Simonen *et al.* 1994), where the C-terminal domain of Hsp150 was exchanged to β-lactamase from *E. coli*. Cell samples (H1065) resuspensed to a fresh prewarmed medium were incubated at restrictive temperatures for 60 minutes before protein translation was blocked by the addition of CHX. Then samples were shifted back to permissive temperatures and incubated for up to four hours. Samples were collected during the incubation and internal, external and cell wall bound β-lactamase activities were measured (I, Fig. 4A). At the restrictive temperature both the cell wall bound and the culture medium activities were very low and stable. Internal activity on the other hand rose slightly even after the addition of CHX. The elevation of activities after the protein synthesis block could be explained by the slower folding of the protein into the mature form under conditions where the ER folding capacity started to be overloaded. After the shift to the permissive temperature, the secretion capacity of the yeast strain started to recover even without new protein synthesis. External activities exceeded internal activities after the 180 minute incubation time point. Thus, the effect of the mutation seemed to be reversible. As a control, we showed also Hsp150ΔBla production from wild type cells at 37 ºC (I, Fig. 4B). Internal activities settled to the basal level after the equilibration of the temperature and external activities rose linearly. We came to the conclusion that the fusion protein was not externalized to culture medium, but remained internal in the *sec13-1* mutant strain under restrictive conditions. The activity measurement did not indicate where the internal form of the protein was localized.

In indirect immunofluorescence microscopy pictures the pattern of the signal of β-lactamase at restrictive temperatures from *sec13-1* cells (H1065; I, Fig. 5c) resembled Kar2p signature (I, Fig. 5a), showing staining around the nucleus and in peripheral ER rims. From *sec18-1* cells a similar staining pattern, although hazier, was seen (H393; I, Fig. 5b) in contrast with scattered staining in *sec7-1* cells (H340; I, Fig. 5d). Microscopic studies therefore suggested that the fusion protein localized in the ER in *sec13-1* cells. We came to the conclusion that Hsp150 fusion protein, Hsp150ΔBla, without C-terminal domain was not able to leave the ER, if COPII functionality was compromised by temperature sensitive mutation in Sec13p.

4.3.2 Processing of the Hsp150 fusion protein SUI-R3-β-lactamase in the *sec13-1* mutant

We also used a short form of the Hsp150ΔBla protein, SUI-R3-β-lactamase (Fig. 12C), where the 11 internal repeats of Hsp150 were reduced to three first repeats and fused to β-lactamase to verify results. Different forms of this protein were easily separated in SDS-PAGE analysis after 15 minute preincubation and pulse chase labeling at restrictive temperatures followed by immunoprecipitation with β-lactamase antiserum. A cytosolic form of the protein was revealed after 30 minute chase from the ER translocation blocking mutant strain *sec63-1* (H1431, I, Fig. 6, lane 13) as a 43.5 kD form in SDS-PAGE. The ER form produced in *sec18-1* migrated in SDS-PAGE as a 63 kD protein (H1432; I, Fig. 6, lane 14). In the *sec7-1* mutant after 60 minute chase (H1433; I, Fig. 6, lane 15) a portion of the protein was in the ER form (63 kD), while some signal migrated as 72 kD and 53 kD forms. The order of protein form processing was clarified by different time points along the chase (not shown). We reasoned that the 72 kD form was a form where numerous glycans were extended to di, tri, tetra and penta-mannoses in the Golgi before Kex2p processing. The 53 kD form was the Kex2p protease processed mature form (R3-β-lactamase) without subunit I. In the *sec13-1* strain we assayed the development of different forms of SUI-R3-β-lactamase during different chase times until 60 minutes and thereafter removed the secretion block by returning the samples to permissive temperatures. In the pulse sample two different forms of the protein were seen (I, Fig. 6, lane 1), a cytoplasmic (43.5 kD) and an initial ER form, less glycosylated than in the *sec18-1* strain after 30 minute chase. As the chase continued only the ER form that slowly achieved more glycans and its electrophoretic migration retarded, was seen in cell lysate samples (lanes 2-4). The estimated size of the ER-form in the anterograde transport mutant after 30 minute chase was larger than the ER form of *sec18-1* strain, due to the functional retrotransport and extended glycosylation, as explained earlier. From culture medium samples no signal was seen under restrictive conditions (lanes 7-10). When samples were returned to permissive temperatures, a faint signal around 53 kD (lanes 5 and 6) was detected from cell lysate and a clear signature from the respective culture medium samples after 30 and 60 minute incubation at permissive temperatures (lane 11 and 12). Thus, SUI-R3-β-lactamase was not transported beyond the ER without functional Sec13p confirming our previous results.

4.3.3 The N-terminal carrier Hsp150Δ

Finally, we assayed the fate of just the N-terminal carrier Hsp150Δ (amino acids 1 - 324) (Fig. 12D) in the *sec13-1* mutant strain, to be sure that the transport defect was not due to the β-lactamase portion under non-functional COPII conditions. Parallel samples were pulse-labeled at 37 ºC and then chased at 24 or 37 ºC for 60 minutes and immunoprecipitated with Hsp150 antiserum before SDS-PAGE analysis. The signal from wild type control cells expressing only the truncated form Hsp150Δ was seen in the culture medium as a 200 kD protein (H430; I, Fig. 7a, lanes 2 and 4), indicating Hsp150Δ transport capability in both temperatures. In the *sec18-1* strain only the ER form (150 kD) of Hsp150Δ was seen under restrictive conditions in cell lysate (H440, I, Fig. 7b, lane 1), while at permissive temperatures both the endogenous and truncated forms

were detected from the culture medium (I, Fig. 7b, lane 4). Since the endogenous form of Hsp150 in the *sec18-1* mutant was not detected in the SDS-PAGE, we showed also the parental strain *sec18-1* (H4) without Hsp150Δ expression in which the Hsp150 ER form (100 kD after one hour chase) was easily seen (I, Fig. 7e, lane 1). For unknown reasons the antiserum recognized only the ER form of the truncated carrier portion, if both proteins were expressed in the same cells. In *sec13-1* cells only the endogenous Hsp150 was transported to culture medium at restrictive temperatures and the truncated form remained internal as expected (H1107; I, Fig. 7c, lane 1 and 2). A similar of result was obtained from another strain expressing only the truncated form of Hsp150. At restrictive temperatures most of the Hsp150Δ protein was internal, and a faint signal was seen in the culture medium compared with the signal at permissive temperatures (H1545; I, Fig. 7d, lanes 1 and 2). Since this strain was obtained after mating and sporulation, it seemed that the fluctuation of the background affected the tightness of the mutant phenotype. Nevertheless, the main conclusion was that the repetitive N-terminal portion of the protein did not contain a determinant for the Sec13p independent ER exit.

4.3.4 The C-terminal domain of Hsp150 harbors a determinant responsible for the Sec13p independent transport

In order to assay the C-terminal domain (Fig. 12E; last 114 amino acids) of Hsp150 in the Sec13 independent ER exit, we fused it to the Hsp150 signal sequence in order to translocate the protein into the ER. We already knew that the Hsp150 signal sequence directed proteins totally posttranslationally to the ER without any flexibility (Paunola *et al.* 1998). Unpublished studies (Suntio and Makarow) indicated that SUI is absolutely needed for the successful translocation of some fusion proteins and is at least beneficial for all the proteins studied. Therefore we fused SUI, including the Kex2p recognization sequence in front of the C-terminus of SUI omitting the repetitive sequence (SUI-Cterm; SUI-CTD). We assayed SUI-CTD transport in the *sec13-1* strain (H1429) at restrictive and permissive temperatures in the same kind of pulse chase experiment as done before, precipitating with the Hsp150 antiserum. At permissive temperatures, as SUI-CTD is expressed under the control of Hsp150 promoter, the expression level is lower and the variably glycosylated protein was not clearly seen in the cell lysate during the pulse, only a very faint veil was detected (I, Fig. 8A, lane 6). After 60 minute chase a clear signal of the processed, Kex2 protease cleaved CTD was seen in the culture medium as a double band around 16 kD (lane 7), resembling CTD produced from control cells in both temperatures (lanes 9 and 11). At restrictive temperatures in the pulsed *sec13-1* strain a smear around 23-29 kD was seen in the cell lysate (lane 2) and the mature Kex2 protease processed form appeared in the culture medium after 60 minute chase (lane 3). Thus, the C-terminus of the Hsp150 protein was secretion competent from yeast cells without Sec13p function, while other marker proteins remained in the ER. Since SUI had no labeling sites (methionines or cysteines), the fate of the N-terminal domain after Kex2p cleavage in the *trans*-Golgi is unknown.

Normally, Kex2p is located in the late Golgi compartment, different than Mnn1p, co-localized with Sec7p (Cunningham and Wickner 1989, Redding *et al*. 1991). Kex2p harbors a tyrosine based localization signal and is shown to cycle between endosomes to relocate into new trans-Golgi-TGN (Wilcox *et al*. 1992, Sipos *et al*. 2004). Our results showed that although ER-exit of several proteins was inhibited in the COPII mutant conditions, the secretion route remained otherwise functional. A separate Kex2p compartment is present based to Kex2p processing of Hsp150 and SUI-CTD also in non functional Sec13p conditions.

To be certain of the role of the C-terminus in Hsp150 transport, we followed another Hsp150 SUI-domain fusion protein where SUII (the whole repetitive region and CTD) was replaced by the β lactamase (H1067; SUI-β-lactamase; SUI-Bla, Fig. 12F), without functional Sec13p. In the pulse chase experiment at restrictive temperatures, the ER form persisted

during the chase time for 90 minutes (I, Fig. 8B) confirming that the N-terminal part of Hsp150 did not cause the COPII independent ER exit.

Thus, the functional Sec13p independent signature that guided ER- exit of Hsp150 in the conditions when other proteins studied remained into ER, locates in the C-terminal domain.

4.3.5 Active determinant in the C-terminus

For eukaryotic cell functionality, an important question is how to keep the cell content organized. Vesicular transport is one key to the order. Whether the ER exit is bulk flow or whether the cargo is concentrated and sorted along the transport route, has been a long ongoing debate as discussed in the introduction. Therefore we studied whether the Sec13p independent exit of Hsp150 could be considered as bulk flow and the fate of other proteins in the *sec13-1* mutant as retention. We fused invertase to the C-terminus of SUI-CTD (Fig. 12G). We assayed its fate in 2% glucose containing medium under the control of the Hsp150 promoter when endogenous secreted invertase production was repressed. At the restrictive temperature, pulse-labeled and 60 minute chased yeast cell samples were immunoprecipitated with the invertase antiserum. Prior to SDS-PAGE analysis, we treated the samples with endoglycosidase H digestion. The treatment removes most N-linked glycans without affecting O-linked glycans. In the secretion competent control cells, the mature form of the fusion protein migrated as a 73 kD protein (H 1540; I, Fig. 9, lane1). In *sec18-1* strain the fusion protein appeared as a 81 kD protein (H1542; ER form; lane 3). The mature form was cleaved by Kex2p. In *sec13-1* mutant strain the fusion protein was mainly Kex2p processed running as a 73 kD protein (H1541; lane 2), while a small portion of the protein remained as 82 kD ER form. As a control we showed the ER form of the induced endogenous endo-H treated invertase migrating as 58 kD protein (H1542; lane 4).

This result indicated that the C-terminus of Hsp150 is actively sorted to exit from the ER and it can also direct a functional Sec13p dependent protein, invertase as a fusion protein to the C-terminus of Hsp150, to the Sec13p independent route. The ER exit of Hsp150 is not bulk flow, but a new flavor of the COPII pathway that may operate also in wild type situations at least when the heat is on. The fact that the ER exit of Hsp150 was much slower in *sec13-1* and not always complete can be explained by ER stress. Accumulation of other biosynthetic proteins inside the ER lumen may affect protein folding and sorting. Retardation of a portion of Hsp150 in the ER form might be due to capture into the normal type of COPII prebudding complexes that were not able to form completely in the absence of Sec13p function.

4.4 ATPase activity on the Hsp150 C-terminal fragment

We next investigated the C-terminal domain of Hsp150 more closely. Aligning the C-terminal domains of the closest PIR-family homologs (II, Fig. 2), we found a Walker A like sequence (GGILTDGKG) in all four family members. The Walker A motif (GGXXXXGKT) was first found in P-loop containing ATPases, and was involved in ATP binding. The Walker B type sequence (hhhhDEXX, where h stands for any hydrophobic residue) was also needed for catalytic activity (Walker *et al*. 1982). A Walker B like sequence was in Hsp150 and its closest homolog Pir1p (II, Fig. 2). To test the ATPase activity, endogenous Hsp150 was isolated from the culture medium (H1642). As a control we used similarly treated culture medium from the Hsp150 deletion strain (H23). The *sec13-1* mutant strain (H1642) was used to block the secretion of other proteins to the culture medium. Hsp150 was concentrated by ultra filtration devices with differential cut-off values. All the protein samples used in ATPase assays were analyzed for impurities by Coomassie staining and Western blotting (II, Fig. 3). To test the potential ATPase activity, 1 µg of the mature glycosylated protein was incubated with α labeled [^{32}P] ATP (0.5 µCi) for 60 minutes. Radiolabeled ATP and ADP were separated from each other by ascending thin layer chromatography (II, Fig. 4A, lane 2). Indeed,

Hsp150 was a Mg^{2+} requiring ATPase. Control reactions done with the *HSP150* deletion strain where an equal volume of the similarly concentrated culture medium was added to the reaction, were not able to hydrolyze ATP (lane 1).

Next we produced the whole Hsp150 protein and the C-terminal domain in *E.coli* as intein fusion proteins (intein-Hsp150, intein-CTD). Fusion proteins were purified by intein-directed binding to chitin resin, and after autoproteolytic induction the target proteins were eluted from the resin. Both fusion proteins, non-glycosylated Hsp150 and CTD had ATPase activity (II, Fig. 4A, lane 4 and 7).

Removing the central part of the ATPase structure by deleting 37 amino acids (CTDΔ; II, Fig. 2 marked with arrows) led to loss of ATPase activity as seen with the intein fusion produced CTDΔ ATPase activity assay (II, Fig. 4A, lane 6). We also made a specific mutation to a conserved lysine (K335) changing it to alanine and purified the mutant form of Hsp150 also as an intein fusion protein. As expected, the ATPase activity was lost (not shown), since the lysine is shown to be responsible for the binding of the γ-phosphate of ATP in the P-loop ATPases (see Saraste *et al.* 1990).

The potential ATPase activities of the three Hsp150 homologs' (Pir1p, Pir3p and Pir4p) C-terminal domains (II, Fig. 2) were also tested. Proteins were cloned as intein fusions and as before, ATPase activities were measured. Only Pir1p harbored ATPase activity, as expected (II, Fig. 4. lanes 8, 9 and 11), since the essential Walker B motif was missing from the Pir3p and Pir4p sequence.

The amount of ADP produced in the reactions was quantified with PhosphoImager (II, Fig. 4B). The highest activity was with the full length non-glycosylated protein, almost twice as much as with the glycosylated yeast-produced authentic culture medium protein. The other nucleotides were analyzed using *E. coli* produced full length Hsp150 protein, but no GDP, CDP or TDP was seen (II, Fig. 5). Thus, Hsp150 was an ATPase and not an unspecific NTPase.

The K_m value for ATP was estimated to be 1.75 μM for the full length non-glycosylated Hsp150 and 1.5 μM for glycosylated Hsp150 and the CTD fragment (II, Fig. 6A). The reaction rate was by Michaelis-Menten equation 14 pmol $min^{-1}\mu g^{-1}$(II, Fig. 6 A). As the steady state turn over rate we calculated 0.53 min^{-1}, which is relatively slow, but compared with unstimulated Hsp70 activity (0.02-0.2 min^{-1}; Flynn *et al.* 1989, Jordan *et al.* 1995), Hsp150 was still more efficient. The conversion of ATP (40 pmol) to ADP and P_i was linear for at least two hours. With non-glycosylated Hsp150 70 % of the ATP was hydrolyzed after two hours (II, Fig. 6B).

4.4.1 Possible function of ATPase activity

4.4.1.1 Role of ATPase outside the cell

Clearly, Hsp150 is an ATPase, but the cellular role of the protein is unknown. The measured ATPase activity was highest with the non-glycosylated whole protein, as if the massive glycosylation that Hsp150 gets along the secretion pathway hinders the ATPase activity. Furthermore, whether ATP would be available outside of the yeast cell is questionable. Yeast has probably evolved as a parasite or at least takes advantage of sugar-full ripening grapes. ATP might be available in those situations in fruit juice, but what is the advantage for yeast of an external ATPase? Heterologous expression of Hsp150 in a pathogenic yeast *Fusarium oxysporum* is reported to protect yeast cells against a tobacco defense mechanism. Plant cells secrete pathogenesis-related proteins to protect plant cells against yeast invasion and one of these proteins, osmotin, induced apoptosis in yeast cells by binding to Pho36p receptor on the plasma membrane. Heterologous expression of Hsp150 in cell wall harboring yeast cells, but not spheroplasts, enhanced protection against osmotin. The first attachment of osmotin to the cell wall happened through an interaction with phosphorus containing glycans (Yun *et al.* 1997, Narasimhan *et al.* 2003, 2005). Perhaps Hsp150 due to the ATP binding activity could mask the phosphate groups by binding to them without hydrolysis. Phosphorylated mannose was probably not a substrate, as Hsp150 is strictly an ATPase.

The Pir-family members are cell wall binding proteins and a portion of Hsp150 can also be extracted from cells as a covalently cell wall bound protein (Kapteyn *et al*. 1999). Previously Sievi and coworkers (2003) have shown that a large portion of non-covalently bound Hsp150Δ (N-terminal carrier of Hsp150, without C-terminal ATPase domain) was released from the cell wall by boiling with SDS. Endogenous Hsp150 is not substantially non-covalently bound to cell wall. However, without ATPase activity a substantial portion of the Hsp150K335A protein was captured by the cell wall and released from the cell wall by SDS-boiling in lysate preparations in wild type secreting cells (II, Fig. 8A). We studied the binding of Hsp150K335A to the cell wall by reducing the phosphorylated glycan content by 90% using a *mnn6* mutant strain (Nakayama *et al.* 1998, Wang *et al.* 1997). We expected that Hsp150K335A binding to the cell wall could be reduced, if there were less binding substrates, but we could not detect any change in Hsp150K335A cell wall association in the *mnn6* strain compared to wild type (Suntio and Makarow, unpublished data). Thus, we were not able to show that the protective role of Hsp150 against osmotin is due to masking of the binding sites.

Other yeasts, such as the human pathogen *Candida glabrata,* secrete several proteins homologous to Hsp150 and at least one (76.8% identity over 99 amino acids) had a similar Walker B and an almost complete Walker A motif as the Hsp150 sequence (UniProt@EBI; Q6FJ79). Whether ATPase activity plays any role in *C. glabrata* pathogenesis remains to be seen. Several different yeast strains harbor various forms of Hsp150 homologs or family members. Also, a remote homology between the Hsp150 C-terminus and *Gibberella zeae* endoxylanase precursor (UniProt@EBI; Q5NDZ1) exists (26% identity over 121 amino acids). Moreover, the Walker A like motif homology existed in the xylanase (GNHVGGKG) as did a potential Walker B motif, suggesting the possibility of a degrative role also for the Hsp150 protein.

The binding of the closest Hsp150 homolog, Pir1p, to the yeast cell wall was shown to concentrate inside the chitin rings in the bud scar (Sumita *et al.* 2005), but Hsp150 bound throughout the cell wall. Furthermore, one repetitive sequence unit is needed for the cell wall association of Pir1p (Ecker *et al.* 2006), and the C-terminal sequence for Pir1p recruitment to bud scars. Clearly, Hsp150 also associates with the cell wall as seen with the ATPase mutant version of the Hsp150 (II, Fig. 8A) and shown in several other studies (Simonen *et al*. 1994, Kapteynn *et al*. 1999, Sievi *et al*. 2003), but numerous wild type molecules of Hsp150 are probably released to the culture medium by its ATPase activity (see section 4.4.1.3.1).

4.4.1.2 Intracellular ATPase activity

If the ATPase activity is not necessary for the externalized form of Hsp150, maybe it is needed inside the cell. Pir1p is needed for Apn1p transport to the mitochondria (Vongsamphanh *et al.* 2001). Apn1p is required for the DNA repair pathway and normally it is transported to the nucleus (Ramotar *et al.* 1991). In the Pir1p deletion strain the mitochondrial DNA suffered about 5 times higher mutation rate. We tried to find interacting partners for the Hsp150 C-terminal domain by two-hybrid interaction fishing. The strongest interacting partners were a nuclear pore complex protein Nup82p, a 2 micron plasmid encoded protein Rep1p (Ahn *et a*l. 1997), and also APC2p (Table 3). Nup82p forms a cytoplasmic side sub-complex with Nup1p and Nup159p, and facilitates Nup116 interaction with the nuclear pore (Fahrenkrog *et al.* 1998, Ho *et al.* 2000). The essential Nup82p function is needed for protein import to and export from the nucleus. Also other nuclear pore components were found after crosslinking in complex with Hsp150, like Nic96p, Nup100p, Nup116p, Nup2p, and Nup60p (Alfarano *et al.* 2005). The direct interaction with the nuclear pore component Nup82p and purification in a complex with nuclear pore proteins supports a possible role for Hsp150 ATPase in the nuclear import of proteins, such as Rep1p. APC2p is a subunit of the Anaphase-Promoting Complex, which is an ubiquitin-protein ligase required for degradation of anaphase inhibitors (Zachariae *et al*. 1998). Although we got two separate hits, the LacZ activity

is very low that might be background or Hsp150 contact also facilitates its transport to nucleus. These interactions were not studied further.

Table 3. Two-hybrid interacting partners with the C-terminal domain of Hsp150 as a bait. LacZ activities measured using Ortho-nitrophenyl-β-D-galactopyranoside as a substrate.

prey	A420nm/ h/10^8cells
Nup82p (aa 394-566)	0.2875
Nup82p (aa 425-567)	0.0637
Rep1p (aa 174-249)	0.0876
APC2p (aa 523-782)	0.0145
APC2p (aa 654-852)	0.0112

4.4.1.3 Role of ATPase activity in protein transport

4.4.1.3.1 Sec24p independent ER exit

Next we studied the role of ATPase activity in the ER exit of Hsp150 in a similar ten minute pulse and 60 minute chase experiment as described before. We assayed the Walker A motif deletion mutant (internal 37 amino acids; Hsp150CTDΔ; Fig. 12H; II, Fig. 1B, Fig. 4), in the endogenous Hsp150 deletion background. Without secretion block the mutant protein was totally mature and partially secreted to the culture medium after a 60 minute chase at 37 ºC (H1872; II, Fig. 7C, lanes 3 and 4). A portion remained bound to the cell wall (lane 4). In the COPII component mutant *sec24-1* that had a similar selective secretion block at restrictive temperatures as *sec13-1,* wild type Hsp150 was externalized to the culture medium. The other biosynthetic cargo proteins studied were totally in their ER forms (Fatal *et al.* 2004). In the *sec24-1* background Hsp150CTDΔ (H1925) at 37 ºC was totally in the internal ER form (II, Fig. 7A, lanes 2 and 4) as in the control strain *sec23-1* at the restrictive temperature (H 2057; II, Fig. 7B, lanes 2 and 4) after a ten minute pulse and a 60 minute chase. Thus, by removing the whole ATPase domain, we lost the Sec24p independent transport.

With the specific point mutation changing the conserved Walker A motif lysine to alanine at the position 335 (Hsp150K335A; Fig. 12I) we further confirmed the essentiality of the ATPase activity for the Sec24p independent ER exit. In control cells (H2264) at 37 ºC Hsp150K335A in a 5 minute pulse sample was mainly in the mature form, only a faint signal could be observed as the 89 kD form that disappeared during the chase (II, Fig. 8A, lanes 2 , 4 and 6). Along the chase a minor portion of the protein was also released to the culture medium (lanes 1, 3 and 5), but the main signal remained as the cell associated mature form. We removed the signal from the cell lysate by treating intact cells with external proteinase K as an indication of the cell wall localization of Hsp150K335A (II, Fig. S2). Thus, the ATPase point mutant was able to fold correctly and exit the ER.

In the *sec24-1* mutant background at permissive temperatures Hsp150K335A was expressed at lower levels, but otherwise behaved similarly as in control cells (H2265; II, Fig. 8B). At restrictive temperatures Hsp150K335A was in a heterogeneously glycosylated ER form, starting from the pulse form that during the chase gained more glycans and retarded in SDS-PAGE, but remained in the ER (II, Fig. 8C), incapable of transport without ATPase activity in this COPII deficient strain. As a positive transport control we compared in the same *sec24-1* mutant background the transport of another Hsp150 point mutant (Hsp150Q400A) at restrictive temperatures. Already after a ten minute chase a faint signature of the protein was seen in the culture medium (H2126; II, Fig. 8D, lane 3). After a 60 minute chase the main portion of the protein was in the culture medium (II, Fig. 8D, lane 5).

We concluded that Hsp150K335A was able to fold correctly at 37 ºC in wild type cells. ATPase activity was needed for functional COPII component Sec24p independent ER exit. Hsp150 detachment from the cell wall seemed to be partially dependent on the functional ATPase.

4.4.1.3.2 ATPase activity in the Sec13p independent ER exit

We used for the *sec13-1* independent transport a shorter version of the Hsp150 protein (SUI-CTD) where the repetitive

region was omitted to demonstrate the essentiality of the C-terminal domain's ATPase activity (Fig. 12E). The ATPase point mutation (K335A) was made to the C-terminus creating SUI-CTDK335A (Fig. 12J). The transport of SUI-CTDK335A was studied at restrictive temperatures in a simple pulse chase experiment performed in *sec24-1* or *sec13-1* mutation carrying yeast strains (II, Fig. 9). In cell lysates in pulse samples SUICTDK335A remained in the ER form, whereas in parallel samples a portion of SUICTD was already in the culture medium as the Kex2p processed form (II, Fig. 9C, lane 2). In the chased lysates SUICTDK335A again gained more glycans, but remained totally in the ER form, as compared with the ATPase activity harboring SUICTD that was in the completely processed mature form in the culture medium. In the *sec24-1* strain the protein behaved similarly. Thus, both of these COPII independent ER exit routes of Hsp150 required the ATPase activity.

4.4.1.3.3 ATPase activity is needed for fast ER exit

In a closer study of the Hsp150 secretion rate (Fig. 14 A and C), after one minute labeling with methionine/cysteine at 37 ºC 67% of the endogenous protein was in the mature form (Fig 14 A lane 1 quantified with PhosphoImager), and an ER-form (89 kD band) represented 23 % of the Hsp150 signal. The rest of the signal came from different Golgi forms (smear between clear bands of 89 kD and 150 kD). Thus, the ER-exit of Hsp150 is even faster than previous estimations supposed. After a one minute additional chase 84 % of the protein was mature and 5.9 % of the signal was still in the ER-form (lane 3). After a two minute chase 92 % of the protein was mature, and only 1.5 % was in the ER-form (lane 5). When the relative amounts of different forms were plotted against time (Fig 14B), the maturation half time was clearly shorter than one minute (the first time point). In similar metabolic one minute labeling the ER form of Hsp150K335A represented 47% of the total after one minute pulse (Fig. 14B lane 1) in an isogenic background. After one minute chase 56% of Hsp150K335A was mature but still 25% of the protein was in the ER form (lane 3). After two minute chase the mature form represented 74 % and the ER form 8 % (lane 5). In calculations from a trend line for Hsp150K335A maturation equation (y=22,353x+9,0091, R^2=0,9891) we extracted the maturation half time of one minute and 50 seconds for Hsp150 K335A (Fig. 14D). Clearly, the loss of ATPase activity retarded the extremely fast ER exit rate of Hsp150 in wild type cells. Whether the retardation was due to protein folding or loss of the transport determinant is not known.

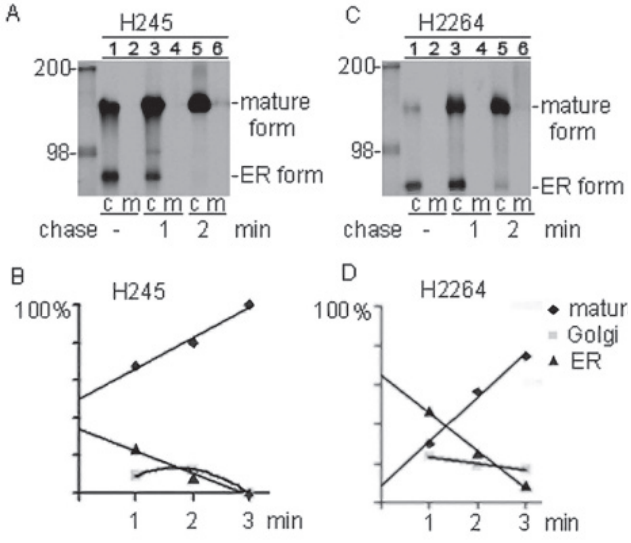

Figure 14. ER-exit and maturation rate of Hsp150 and Hsp150K335A. Normal cells (A; H245) and isogenic background expressing Hsp150K335A (C; H2264) were preincubated for 15 minutes, pulse-labeled with 100 µCi [^{35}S]-methionine/cysteine for 1 minute (lanes 1 and 2), and chased for 1 minute (lanes 3 and 4) or 2 minutes (lanes 5 and 6) at 37 ºC. The cell lysate (c) and respective medium samples (m) were immunoprecipitated with Hsp150 antiserum followed by SDS-PAGE. Protein size standards (kDa) are indicated on left. Signals were evaluated with Phosphorimager and relative amounts were plotted against time (B; Hsp150, D; Hsp150K335A).

The different route of Hsp150 from the ER to the exterior of the cell is obvious if the transport rates of other proteins from the ER to the protein's functional location are compared. Losev and coworkers (2006) estimated that the half time transport rate from the ER exit through the Golgi is approximately seven minutes in wild type cells for the common transport markers α factor and CPY. After one minute metabolic labeling α factor was in the partially core glycosylated ER form, but also a diffuse Golgi form was seen. They estimated the half time of the ER exit to be one minute for α factor. Transport through Golgi was estimated to last for 4-5 minutes and the maturation half time of the protein was around 7 minutes. With CPY, they estimated the *intra*-Golgi stage to take approximately the same time. They stated that the equal transport rate is one proof of the cisternal maturation theory. As a second proof, they showed that GFP tagged *cis* Golgi protein localization was gradually replaced by a later occurring Golgi protein in a single Golgi unit. The Golgi cisternal maturational change from the beginning to the end took around seven minutes. The maturation rate of Hsp150 in wild type cells is much faster: after one minute labeling 76 % of the protein had left the ER and 67% of the protein was already in the mature form. Estimated protein maturation half time is less than one minute, arguing for a different route for the Hsp150 transport than the Golgi cisternal maturation route. Also the mutant form was transported after the ER-exit through Golgi complex faster than the cisternal maturation route supposed. It might be that fast ER exit is achieved with ATPase activity, but transport through Golgi is dictated by other part of the protein.

4.4.2 Possibilities, how ATPase activity could direct Hsp150 to coat structures

This "fast track transport route" probably starts at the level of the exit from the ER. It uses a partially conventional transport pathway, as many early secretion pathway mutants like *sec23-1*, *sec12-4*, *sec31-2*, *sec16-1* (Suntio and Makarow, unpublished result), *sec22-1* (Gaynor and Emr 1997) and *sec18-1* completely block Hsp150 transport. However, anterograde v-SNARE Bet1p (Gaynor and Emr 1997) or GTPase Ypt1p (H264; Fig. 15, left panel) function was not necessary, although these proteins were essential for yeast growth and for the ER exit of common cargo protein.

Sfb2p (Sec24Bp) overexpression can suppress *SEC24* deletion and the protein can replace Sec24p in vesicle formation (Peng *et al.* 1999) depending on the yeast background (Peng *et al.* 2000). From a triple mutant SEC24 (*sec24-1*, *sfb2Δ*, *sfb3Δ;* Karhinen *et al.* 2005) strain externalization of Hsp150 to the culture medium was inefficient but still selective. From double mutants (*sec24-1 sfb2Δ* or *sec24-1 sfb3Δ*) Hsp150 secretion kinetics were like from the single mutant *sec24-1* (Fatal *et al.* 2004, Karhinen *et al.* 2005). Since Sfb3p is shown to be disabled in SNARE binding (Miller *et al.* 2003), in the double mutant *sec24-1 Δsfb2* either a mutant form of Sec24p (a C-terminal variant of Sec24p; Karhinen *et al.* 2005) or an unknown component is responsible for SNARE inclusion to Hsp150 transport containers.

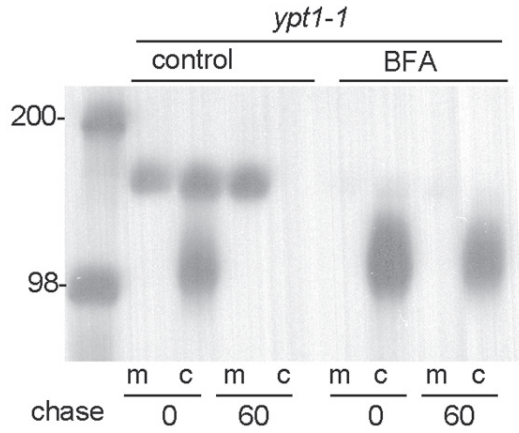

Figure 15. Endogenous Hsp150 secretion in *ypt1-1* temperature sensitive mutant strain (H264) after 15 minute preincubation at restrictive temperature, followed by 5 minute metabolic labeling and CHX chase for 60 minutes. Samples were immunoprecipitated with Hsp150 antiserum before SDS-PAGE. Control reactions were performed by adding 10 µl of ethanol. BFA (10 mg/ml dissolved in ethanol) was added 10 minutes before labeling to growth media. c; cell lysate sample, m; culture medium sample. Protein size standards are indicated on the left.

How ATPase activity directed Hsp150 to coat structures that lacked functional Sec24p and Sec13p, but contained Sec23p and Sec31p, is not known. Either the mutated non-functional COPII proteins could support the ER exit of Hsp150 or they were replaced by homologs as results of Karhinen and Makarow (2005) suggest. Possibly the nuclear pore complex and coat complexes evolved from the same origin as discussed above. Sec23p was found to interact with Nup42p as well as Nup116p. Nup116p was affinity captured with Sec13p and Seh1p (Allen *et al.* 2001). Nup116 was found also to interact with the v-SNARE Bos1p by two-hybrid screen (Ito *et al.* 2001). Maybe some of these interactions could support the ER exit of Hsp150 under the non-functional COPII coat conditions.

Could the phosphate binding Walker A motif of Hsp150 ATPases bind directly to the phosphorus group of a lipid, such as phosphatidylinositol 4-phosphate or phosphatic acid? If it binds, it still would need to communicate to the cytosolic side of the membrane to the forming coat. One possibility is a specific type of lipid raft formation that in the ER lumen binds Hsp150 excluding other proteins and on the cytosolic side binds coat proteins. This intriguing possibility is less obvious, since phosphate binding is not sufficient but the ER exit of Hsp150 needed ATP hydrolysis. Mutation of the Walker A lysine of MRD1 gene to alanine affected protein function but not binding to a photoactivated ATP homolog (Azzaria *et al.* 1989) suggesting that Hsp150K335A was still capable of binding ATP.

Most certainly a transmembrane receptor is needed for the specific capturing of the soluble glycoprotein Hsp150 from the ER mixture containing hundreds of different proteins blocked due to the non-function COPII coat. As a transcriptionally heat inducible (*Saccaromyces* genome database; http://www.yeastgenome.org/), ATPase activity containing protein Hsp150 could be a chaperone that binds to unfolded proteins or hydrophobic peptides. Any hydrophobic peptide could serve as a binding domain, but as a receptor, it should also be a transmembrane protein interacting with the coat. The binding of any other peptide except SUI (Russo *et al.* 1992) and the fore-mentioned soluble proteins to the C-terminus has not been noted. Chaperones such as Kar2p contain two different domains: an ATP binding domain and a peptide binding domain. Whether Hsp150 is a chaperone and has a peptide binding domain, which is used also to bind to transport receptors remains to be seen. Maybe ATPase activity is connected to putative receptor protein activation through phosphorylation that then recruits an unconventional coat structure leading to Hsp150 containing transport vesicle formation.

Summing up, we state that Hsp150 is selectively transported out of the ER in vesicles with a partially different or temperature sensitive form of the COPII coat with the aid of C-terminal ATPase activity. Thereafter protein is transported through Golgi complex using approximately ten times faster strategy than the cisternal maturation route in yeast.

4.5 Retrotransport operated by COPI had exceptions

COPII coat functions unquestionably at the ER exit but the role of the COPI coat in the ER exit has evoked variable opinions. In yeast, it is suggested to function at least in retrotransport and by blocking retrotransport a secondary defect to anterograde transport is induced (reviewed in Pelham 1994, Gaynor and Emr 1997). Gaynor and Emr (1997) showed that a subset of medium or periplasmic proteins, such as invertase and Hsp150 were transported to the exterior of the cell as hypoglycosylated versions in *sec21* mutants. Hsp150 was externalized from all the retrotransport mutants tested (*sec21-3*, *ret2-1*, *sec33-1*, *ret3-1*, *sec27-1* and *ret1-1*; Gaynor and Emr 1997). We studied further which portion of the Hsp150 protein harbored the COPI independent transport determinant.

4.5.1 The N-terminal fragment in COPI independent transport

First we looked if the C-terminal domain was essential for the COPI independent transport using Hsp150Δ-β-lactamase (Hsp150ΔBla;

Fig. 12B) where the C-terminal domain was replaced by β-lactamase. We studied protein secretion for two hours under restrictive conditions separating internal and external lactamase activities in the *sec21-3* strain (H786) shown to block retrotransport, and compared those with the wild type strain (H675) and anterograde ER exit blocking strains *sec18-1* (H393) and *sec23-1* (H484) (III, Fig. 2). External β-lactamase activity increased linearly during the incubation time in the *sec21-3* and the wild type strain. Internal activity accumulated slightly in *sec21-3* cell lysate samples, while in ER exit blocking mutants internal activity increased linearly at the beginning of the incubation at restrictive temperatures. After one hour especially in the *sec23-1* strain activity accumulation slowed down. Culture medium activities were close to the baseline in *sec18-1* and *sec23-1* strains.

Thus, Hsp150ΔBla secretion continued from the COPI mutant cells to the culture medium almost like in the wild type strain. The mutation did not affect the capacity to synthesize or transport this Hsp150 variant during the two hour incubation.

The activity results were verified by pulse chase experiments in *sec21-3* cells both with 30 minute and 60 minute preincubation at the restrictive temperature before a ten minute metabolic labeling and immunoprecipitation with β-lactamase antiserum followed by SDS-PAGE analysis (III, Fig. 3). Hsp150ΔBla was transported to the culture medium completely after a ten minute chase, if the preincubation time was short (III, Fig. 3A, lane 3). Internal forms of 110 kD and 66 kD were seen in the ten minute pulse lysate (lane 2). Based on control strains *sec18-1* and *kar2-159*, apparently these forms represented ER and cytosolic forms. Hsp150ΔBla produced after the 60 minute preincubation time was externalized to the culture medium mostly after a ten minute chase (III, Fig. 3B lane 3), only minor signal was cell associated (lane 4) and internal forms were completely abolished after an 80 minute chase (lane 10).

We also studied Hsp150 N-terminal fusion to a neural growth factor ($Hsp150\Delta NGFR_e$) that had potential to form 12 sulfhydryl bridges and folds to an elongated form compared with the globular β-lactamase. We used another allele of *SEC21*, *sec21-1* that was originally isolated as an anterograde transport blocking mutant and showed also the retrotransport defect (Hosobuchi *et al.* 1992). All the Hsp150 proteins tested, Hsp150 (III, Fig. 5Ab, H830), Hsp150ΔBla (III, Fig. 5Ac, H831) and $Hsp150\Delta NGFR_e$ (III, Fig. 5Aa, H834), were transported to the culture medium after the ten minute preincubation, metabolic labeling and the 30 minute chase. With α 1,6-mannose extension recognizing antiserum from the culture media no other N-glycosylated proteins except $Hsp150\Delta NGFR_e$ were detected (III, Fig. 5Ba, lane 3). The antiserum recognized from the culture medium O-mannosylated Hsp150 versions due to lack of competition. In cell lysates various N-glycosylated proteins accumulated and precipitated as an aggregated smear. From culture media samples, we could conclude that endogenous Hsp150 was the first to be secreted and fusion protein transport was slower (III, Fig. 5Ba, lane 1). Furthermore, the N-terminal fragment was sufficient to escort heterologous proteins out of the yeast ER under conditions when COPI directed retrotransport was non-functional.

The non-functionality of the retrotransport in *sec21-1* (H235) was confirmed by metabolic labeling and immunoprecipitation with CPY-antiserum. CPY was retarded in the ER under the very same conditions when Hsp150 was externalized to the culture medium. The p1 form of CPY was prominent after a 30 minute chase at restrictive temperatures (III, Fig. 5C, lane 2) while at permissive temperatures the protein was totally mature (lane 4).

4.5.2 The amount of repeats

A shorter version of the Hsp150Δ-β-lactamase was made by truncating the repetitive portion of the protein to the four first repeats and joining it to β-lactamase (Hsp150-R4-Bla; Fig. 12K). In control cells (H791) without any secretion mutation at 37 ºC this protein was transported with somewhat slower kinetics to the culture

medium than the 11 repeat-containing Hsp150Δ-β-lactamase. After 30 minute preincubation and five minute pulse-labeling, followed by anti β-lactamase immunoprecipitation and SDS-PAGE analysis, a 67 kD ER form was seen in cell lysates with a cytosolic 50 kD form (III, Fig. 4B, lane 2; ER form was verified in the *sec18-1* strain; Fig. 4A, lane 13; H827). These forms were processed into a mature form (78 kD) almost completely after a ten minute chase (lanes 5 and 6). A portion was released to the culture medium and a portion was released by SDS from the cell wall in lysate preparations. In the *sec21-3* mutant (H792) Hsp150-R4-Bla protein transport in a similar pulse chase experiment was slower than in the wild type cells (III, Fig. 4A). The first sign of the protein in the culture medium samples was seen after a ten minute chase (III, Fig. 4A, lane 5) and the protein was completely mature after a 40 minute chase (lanes 9 and 10). Thus, even the shorter version of the Hsp150 protein was capable of leaving the ER without functional retrotransport operated by the COPI coat, although eleven repeats allowed the protein to fold inside the ER lumen more quickly, or alternatively the COPI independent transport determinants were displayed better in the longer protein.

4.5.3 SUI transport is defective without COPI operated retrotransport

The influence of SUI on the COPI independent transport was studied by using a version of Hsp150 where SUII was omitted by joining β-lactamase directly to SUI (SUI-Bla; Fig. 12F) under the control of Hsp150 promoter. From *Sec21* alleles, we selected *sec21-1* (H842) since in our experiments it had a more severe defect in CPY transport than *sec21-3*. Both mutants were thermosensitive and had a growth defect at restrictive temperatures. SUI-Bla transport was characterized first by activity assays in different strains (III, Fig. 6). In wild type cells (H839) at 37 ºC β-lactamase activities were quite low, almost 80 % lower than Hsp150Δ-β-lactamase activities, but the internal activity did not accumulate and the culture medium activity slowly elevated (III, Fig. 6A). In the *sec18-1* strain (H840) the activity accumulated internally, as studied from samples taken with 30 minute intervals for up to 2 hours (III, Fig. 6B). In the *sec21-1* strain the activity rose inside the cells, and external activity was like in *sec18-1* strain, close to baseline. Still those same mutant *sec21-1* cells in parallel samples after invertase promoter derepression were able to externalize invertase activities (III, Fig. 6D).

In metabolic labeling and immunoprecipitation experiments done with SUI-Bla marker protein in wild type cells, we noticed a strong signal of 46 kD in the cell lysates after a three minute pulse (III, Fig. 7A, lane 2) that mostly disappeared during a 60 minute chase at 37 ºC (lane 4). The same was seen, if cells were shifted to permissive temperatures; only a trace of the mature protein of 52 kD could be detected in the cell lysates and the culture medium (lanes 5 and 6). In the *pep4Δ* strain (H911) where vacuolar degradation functions were defective, the mature marker protein signal was present in substantial amounts after a 60 minute chase in the cell lysate (lane 7). In the *sec21-1* strain the ER form of SUI-Bla was persistent during a 60 minute chase at the restrictive temperature (III, Fig. 7 B, lane 4). No signal was seen in the culture medium (lane 3). If the 60 minute chase was done at permissive temperatures, the signal of the protein almost completely vanished, and only a trace was seen in the culture medium (lane 5) and cell lysates (lane 6). The signal in the *sec18-1* strain was quite constant during the chase at restrictive temperatures (lane 8).

These results indicated that SUI-Bla, although in its catalytically active form, was recognized by the cell quality control mechanisms in the Golgi or was marked already in the ER for the vacuolar degradation route. If the marker protein's ER exit was prohibited, its signal was fairly stable. It remained in the active form inside the ER lumen in the *sec18-1* and *sec21-1* mutants and was not substantially targeted to cytosolic degradation machineries.

We verified the vacuolar degradation by protein localization studies with immunofluorescence microscopy. Cell samples were incubated for 30 minutes at

37 ºC, chased with CHX for 30 minutes to allow the proteins to settle in their transport end point in different mutant strains, fixed and stained with β-lactamase and secondary rhodamin-conjugated antibodies. The stain concentrated in internal round ring structures that co-localized with the central vacuole in the Nomarski-optic pictures in the *pep4Δ* strain (III, Fig. 8, panel A1 and A2). In the *sec18-1* or in the *sec21-1* strain (panels B1 and C1) the signal did not resemble vacuolar staining; it was a hazy signal coming from internal membranes, sometimes around the nucleus, as the ER staining should. In *sec21-1* the signal was even more punctuated, resembling Bip-body like staining that is commonly seen when protein transport from the ER is prevented (Nishikawa *et al.* 1994). The Bip bodies also concentrate proteins that are targeted to degradation (Sullivan *et al.* 2003).

Immunofluorescence based protein localization studies confirmed that when the secretion pathway was functional, SUI-Bla was targeted to the vacuole. In the *sec21-1* strain under restrictive conditions it most probably localized inside the ER. Altogether, we were convinced that SUI did not contain determinants for the COPI independent transport.

4.5.4 Role of repetitive units in SUII

To pinpoint the determinant for the COPI independent transport we ensured that repetitive units without SUI were able to exit the ER in the strict mutant *sec21-1* under restrictive conditions. We removed SUI, joining the signal peptide directly to SUII in the β-lactamase containing construct forming SUII-β-lactamase (Fig. 12L) and assayed protein transport by activity in wild type (H890) and *sec21-1* strain (H891) at 37 ºC. Internal activities were high already at the beginning of the experiment, because the protein had difficulties in the post-translational translocation into the ER lumen without SUI (not shown). Paunola and coworkers (1998) have shown that β-lactamase portions fold to enzymatically active form already before translocation into the ER. In Hsp150ΔBla translocation, the cytosolic fold was at least partially unfolded into linear chain that then in the ER lumen was refolded again (Paunola *et al.* 2001). We used CHX chase to study protein transport with this slowly translocating marker protein. CHX was added simultaneously to cell samples when cells were shifted to pre-warmed (37ºC) fresh culture medium. Samples were collected for up to six hours and external and internal activities were

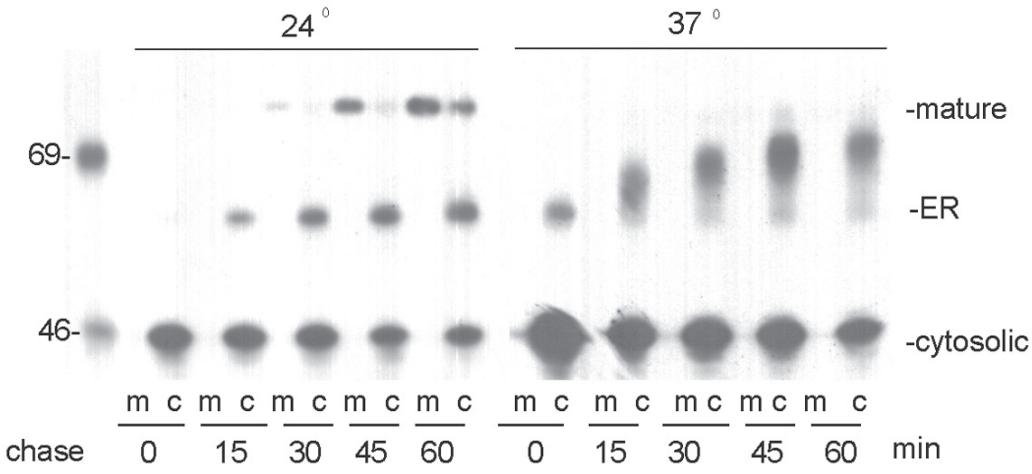

Figure 16. R6-bla secretion in the COPI mutant strain *sec21-1* (H1359) at permissive and restrictive temperature. Cell samples were preincubated for 15 minutes at the indicated temperatures before metabolic labeling, CHX chase and immunoprecipitation with Bla antiserum before SDS-PAGE. Chase times are indicated below the samples. c; cell lysate sample, m; culture medium sample. Protein size standards (kDa) are indicated on the left.

measured. In the wild type as well in the *sec21-1* mutant strain internal activities started to slowly diminish and external activities increased with similar relative kinetics in both strains (III, Fig. 9).

A similar protein where the amount of the repeats was reduced to the last six (signal peptide was joined to amino acid 187 in the Hsp150 sequence and C-terminus was replaced by β-lactamase; R6-Bla Fig. 12M) showed also ER translocation problems as expected. However, it suffered also ER exit problems. 69 % of the protein after one hour chase in the metabolic labeling and immunoprecipitation experiment was cytosolic, 30 % in the ER form and <1% was mature under restrictive conditions assayed in the *sec21-1* strain (Fig. 16). After a two hour chase the amount of the mature signal was not increased (not shown). Under permissive conditions substantial amounts of the protein were mature after one hour chase (Fig. 16). With intact eleven repetitive units (SUII-Bla) the percentage amount of the externalized culture medium activity was already substantial after two hour incubation at the restrictive temperature and was linearly increasing, which was not the case for the six repeat harboring protein in the *sec21-1* mutant. In metabolic labeling and immunoprecipitation experiments, no internal ER form of SUII-Bla accumulated in the *sec21-1* strain, and only the cytosolic and the culture medium located forms could be detected (Suntio and Makarow, unpublished data; not shown). This suggested to us that the N-terminal repetitive part of the protein harbored a special feature capable of ER exit without the functional COPI coat. Alternatively, protein folding was affected so that the COPI independent signature was not presented properly by the six last repeats in the truncated protein.

4.5.5 O-glycans in cargo selection

We analyzed by MALDI-TOF the glycan content variation of both SUII and SUI. A histidine tagged version of the Hsp150 protein was isolated from the culture medium (H849) and purified gently in a nickel column, whereafter SUI and SUII were separated from each other by reverse phase chromatography and subjected to mass spectrometry to analyze the size variation. We knew from our previous work after β elimination and thin layer chromatography that Hsp150 SUII contained mostly di-mannoses but also tri-, tetra- and penta-mannoses in a ratio of 4:1:1:1 (Jämsä *et al.* 1994). Seven glycans with this ratio have a joint mass of 3240 kD (20 times mannose mass 162 daltons). In mass spectrometry SUII was sized between 60000 and 70000 daltons, the average being 64668 daltons (III, Fig. 10A). The mass of the SUII polypeptide is estimated to be 34483 daltons without glycans, leading to approximately 63-85 glycosylated serines or threonines of the potential 85 acceptor residues in the ER lumen. The expected mass of SUI is 5375 daltons, and time of flight analysis gave a molecular mass variation from 9674 to 13528 daltons (III, Fig. 10B), meaning that the glycan content varied between 26-50 mannoses (4299-8153 daltons) in the mature SUI. The mannose chain length or quantity in mature SUI is not known, but it is obvious that several of the 21 potential sites are mannosylated in the ER lumen. If Golgi glycan extensions to SUI were of the same kind as in SUII, this would yield 10-20 occupied glycan acceptors in the ER lumen. Therefore, it seemed that the overall amount of glycosylation initiation was not the determinant in the repetitive units for COPI independent transport.

4.5.6 Putative receptors?

We had mapped the COPI independent determinant to the repetitive portion of Hsp150. The determinant was an active selective unit since it guided heterologous fusion proteins out of the ER. Since COPI independent transport was not a bulk flow and the model protein was totally soluble it most certainly also needed a transmembrane protein that aids the packing to normally composed COPII vesicles. The known two-hybrid interactions to Hsp150 from databases (Alfrano *et al*. 2005; Ybr064w, Ydl023c, Ybl044w and Ynl321w) were studied by activity after transforming Hsp150ΔBla to deletion strains obtained from Euroscarf, but no clear retardation in transport was observed (Suntio and Makarow, unpublished results, not shown).

Crossing these strains with the *sec21-1* mutant or crossing to COPII deficient strains and selecting haploid double mutants did not change the secretion of Hsp150ΔBla at restrictive temperatures (Suntio and Makarow, unpublished results, not shown). The only effect observed was a tenfold enhanced expression of Hsp150ΔBla in stationary phase cells caused by YBR064w overexpression (Suntio and Makarow, unpublished data, not shown). These deletion strains did not affect COPI independent transport.

We speculated that the receptor might recognize a specific glycan moiety, and therefore we studied Hsp150Δ-β-lactamase transport in the *EMP47* deletion strain. Emp47p harbors homology towards lectins and its cytosolic C-terminal tail is shown to bind directly to COPII components (Sato and Nakano 2002, 2004). Sato and Nakano (2004) reported that a protein from the culture medium of around 150 kD (probably Hsp150), migrated in the SDS-PAGE gel in a less glycosylated form in the *EMP47* deletion strain as compared to control cells. We studied Hsp150Δ-β-lactamase transport in the *EMP47* deletion strain (H1801) by metabolic labeling and immunoprecipitation. Fusion protein transport was unaffected excluding the possibility that the Emp46/47p complex is the only receptor responsible for the transport of the repetitive portion of Hsp150 (Fig. 17 compared to previous result, for example Paunola *et al*. 1998). At least in this functional COPI transport strain, *EMP47* deletion did not substantially affect Hsp150Δ-β-lactamase transport kinetics.

Although we did not find the receptor responsible for COPI independent transport we were convinced of its existence. There may still be two different determinants in the area of the four first repeats and both may be responsible for the COPI independent transport. The possibilities include a certain type of O-glycosylation and flanking areas between repeats. We propose that the COPI independent transport signature might reside in the flanking areas between the repeats simply because similar repeats, the six last ones, did not support the COPI independent ER exit. These determinants, specific glycosylation and flanking areas, may form a joint determinant like in the case of ERGIC-53 recognition. ERGIC-53 was recognized through the glycan moiety and through a β turn in the peptide chain, and both were equally important for ER exit (Appenzeller-Herzog *et al.* 2005).

The diversity of COPI retrotransport independent receptors is supported by the notion that COPI independently transported proteins Hsp150, invertase and Bgl2p do not have a lot of sequence similarity. Bgl2p was discovered after protein isolation from the culture medium samples of *sec21-1* cells grown under restrictive conditions, followed by peptide trypsination and Matrix Assisted Laser Desorption /Ionization- Time Of Flight (Suntio and Makarow, unpublished results, not shown). Hsp150 is an O-mannosylated protein, whereas invertase and Bgl2p are N-glycosylated (Esmon *et al*. 1987, Mrsa *et al*. 1993). Probably, these proteins are recognized by different receptors that all are COPI independent. If there is one single receptor for COPI independent transport that would recognize all three proteins, then the recognition element could be a three-dimensional structure.

In the known transport receptor *ERV29* deletion strain α factor transport rate *in vivo* is retarded but transport is not totally blocked (45 % was externalized after a 20

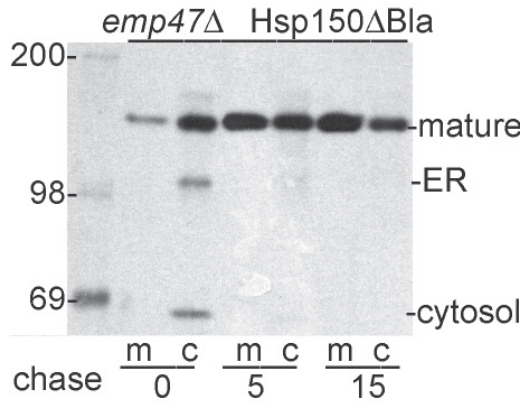

Figure 17. Hsp150Δ-Bla secretion in *emp47*Δ strain (H1801). Cell samples were preincubated for 15 minutes before 5 minute metabolic labelling, CHX chase and immunoprecipitation with Bla antiserum. Different chase times are indicated below.the samples. c; cell lysate sample, m; culture medium sample. Protein size standards (kDa) are indicated on the left.

minute chase; Belden and Barlowe 2001b). This may indicate multiple receptors for α factor as well, or then the secretion without the receptor is bulk flow. In *in vitro* assays α factor transport was completely blocked in the ERV29 deletion strain. The concept of bulk flow would allow that all proteins are inserted into transport vesicles also in situations of non-functional COPI retrotransport, which is quite opposite to what is observed. In the non-functional *sec21-3* strain α factor remained in the ER (Gaynor and Emr 1997). A second possibility is that the COPI coat is directly needed by a subset of proteins to complete the ER exit in the anterograde transport.

4.5.7 Other retrotransport routes than COPI?

Since Hsp150 transport was selective under non-functional COPI coat conditions for up to three hours and thereafter also protein synthesis was affected (not shown), there must be another retrotransport route, for example for SNARE protein retrieval. Hsp150 transport was also dependent on SNAREs such as Sec22p (Gaynor and Emr 1997) and totally blocked in the *sec18-1* mutant, indicating a SNARE assisted membrane fusion step in the ER exit which could not be overcome by new protein synthesis. In mammalian cells the existence of a Rab6 regulated retrotransport route was revealed by studying Shiga toxin transport to the ER (Giroid *et al.*1999). Yeast Rab6 homolog *YPT6* mutant indicated that Ypt6p functions in endosome-to-Golgi transport, in the Golgi retrograde transport, and possibly also in Golgi-to-ER trafficking (Luo and Gallwitz 2003). The authors showed that the combination of *ypt6-2* and *sec21-1* had a more severe growth defect than a single mutant. We crossed the temperature sensitive mutants *ypt6* and *sec21-1* and picked up after sporulation a double mutant haploid strain and followed Hsp150ΔBla transport at restrictive temperatures. In the temperature sensitive *YPT6* strain Hsp150ΔBla was secreted to the culture medium in the metabolic labeling and immunoprecipitation experiment in two different forms, as a mature form and a form above the mature form that was the non-cleaved form of the protein, glycans fully extended (Fig. 18, lane 1 and 2). Clearly, Ypt6p^{ts} affected endosome-Golgi transport, either misplacing Kex2p or exporting a portion of Hsp150ΔBla from an earlier compartment. In the double mutant *ypt6*^{ts} *sec21-1* Hsp150ΔBla secretion pattern was the same (lanes 3 and 4) as in the single *ypt6*^{ts} mutant indicating that Ypt6p regulated retrotransport was not at least solely responsible for retrieval that allowed the continued secretion of Hsp150 in COPI coat deficient conditions.

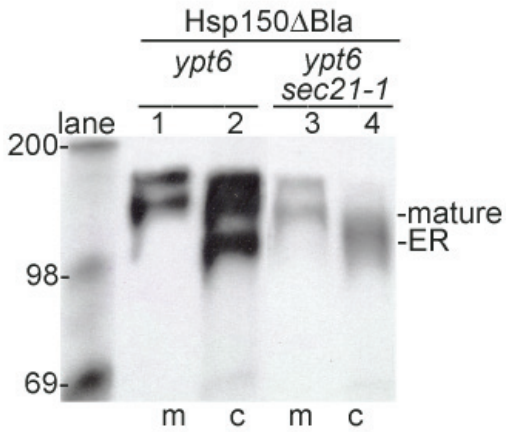

Figure 18. Hsp150Δ-Bla secretion in *ypt6*^{ts} (H1920*)* and *ypt6*^{ts} *sec21-1* (H1890) strains after 15 minute preincubation, 5 minute metabolic labeling and 60 minute CHX chase, followed by immunoprecipitation with Bla antiserum. c; cell lysate sample, m; culture medium sample. Protein size standards are indicated on the left.

4.5.8 Fate of Golgi markers in COPI mutants

Proteins that are transported to their destination without the functional COPI coat are substantially hypo-glycosylated. Hsp150 in the culture medium runs as a 145 kD protein and invertase that normally is heterogenously glycosylated (100-200 kD) was externalized as a 100 kD protein from the *sec21-3* strain (Gaynor and Emr 1997) indicating that the Golgi is not totally functional. A fraction of the hypo-glycosylated invertase contained α 1,6- and α 1,3-linked mannosyl extensions (Gaynor and Emr 1997) that are indicators for Golgi

passage. What happened to the Golgi in the COPI mutant conditions? In the *sec21-3* mutant Gaynor and Emr (1997) followed the location of an early *cis*-Golgi transferase Och1p by immunofluorescence. After a 30 minute incubation at the restrictive temperatures they saw the punctate localization changing to a more diffuse staining that was not ER staining. They explained the origin to be tubulo-vesicular structures. Staining of *trans*-Golgi marker Mnn1p was unaffected. Abe and coworkers (2004) studied GDP-mannose transporter (GMT) localization to Golgi in different secretion mutants and documented its localization to the ER in the *sec23-1* mutant, but in the COPI mutant to the vacuole. So, GMT seemed to recycle between the ER and Golgi and if the recycling was blocked by the COPI mutation, the membrane flow eventually targeted the protein to the vacuole. Rer1p, a Golgi located membrane protein, was reported to experience the same fate (Sato *et al.* 2001). Sec22-myc-α chimeric proteins reached the Kex2p compartment in the non-functional COPI conditions and were cut by the protease. In normal conditions Sec22p recycled between the ER and Golgi and the chimera was mainly uncut (Ballensiefen *et al.* 1998). In an *arf1-3* temperature sensitive mutant (*ARF1Δ, ARF2Δ* strain background) Mnn1p localized to unusual ring-like structures, separate from the vacuole (Gaynor *et al.*1998) suggesting that some kind of separate Golgi-like complex existed in the non-functional retrotransport conditions. Todorow and coworkers (2000) studying an elongating α 1,6 mannosyl transferase complex showed it to locate in the vacuole if retrotransport to the ER was blocked.

Perhaps the question in the COPI independent transport was what kind of vesicles were able to fuse to this Golgi remnant, a pre-vacuolar compartment or even the vacuole? Clearly, Hsp150 was transported through this compartment; the culture medium form of the protein was Kex2p cleaved (e.g. R3-Bla, SUI-CTD; Suntio and Makarow, unpublished results). In plant seed storage protein accumulation, one transport route starts directly from the ER to a storage vacuole (Herman and Schmidt 2004). The only known direct transport route from the yeast ER to the pre-vacuolar compartments or to the vacuole is autophagosome formation. Autophagosome formation is induced by ER stress (Yorimitsu *et al.* 2006). Since Hsp150 transport was dependent on Sec31p but autophagosome formation was not, the normal type of COPII vesicles were needed for the COPI independent transport of Hsp150 to the Golgi remnant. The repetitive units of Hsp150 SUII were not able to support COPII component Sec13p and Sec24p independent transport. Thus, for these constructs the normal type of COPII components formed the vesicles and Hsp150 transport continued for several hours to the exterior of the cell suggesting another type of retrieval of essential components than COPI. It is possible that all anterograde directed proteins as well as their receptors were directed with the membrane flow to vacuole or prevacuolar compartment and some, if not all receptors, are targeted back to ER with this additional retrotransport route, raising an open question, why the ER exit of some proteins is blocked.

4.6 Possible role of the COPI primer ARF in anterograde transport vesicle formation

4.6.1 Hsp150 is maturated in non-functional ARF GEF Sec7p strain

Some of the COPI coat components were in the first place isolated as anterograde transport blocking mutants and later discovered to affect also retrotransport (Hosobuchi *et al.* 1992, Letourneur *et al.* 1994). The recruitment of COPI coatomer on the membrane is initiated by the spatial signal of activated ARF-family members (see Donaldson and Honda 2005). From the inactive cytosolic pool ARFs are targeted to the membrane and firm binding is achieved after GTP binding to ARF that is preceded by GDP dissociation accomplished by a GEF. In the yeast early transport pathway Gea1p, Gea2p and Sec7p are reported to function as ARF GEFs (see Jackson and Casanova 2000). Hsp150 was externalized to the culture medium in the double mutant *gea1-19 gea2Δ* (Peyroche *et al.* 2001) and

our results showed Hsp150 maturation in the *sec7-1* strain (Fig. 13, lane 8) as already explained. Hsp150 was totally mature already after the pulse (not shown), but remained intracellular.

4.6.2 The effect of BFA on Hsp150 transport

4.6.2.1 BFA in wild type cells

As Hsp150 was transported out of the ER and became mature in the *SEC7* mutant as well as in the *GEA* double mutant, we studied the effect of BFA on Hsp150 transport. All yeast GEFs on the early transport pathway are reported to be sensitive to BFA (Peyroche *et al.* 1999). Since Hsp150 is transported out of the cells without the functional COPI coat it was plausible that Hsp150 would be transported out of the ER after BFA treatment and would at least develop to the mature cell associated internal form. Gaynor and Emr (1997) studied the effect of BFA on Hsp150 transport and reported that Hsp150 was Golgi modified and thus blocked to Golgi in BFA treated cells. Furthermore, they based their interpretation partially on the assumption that Golgi enzymes were reported not to merge to the ER by BFA treatment in yeast (Graham *et al.* 1993). We repeated the pulse-chase labelling in the presence of pre-added BFA. At 37ºC the hypo-glycosylated ER form of Hsp150 persisted during a 30 minute chase (Fig. 19A lane 2). Occasionally the effect of BFA was lost during a prolonged chase (60 minutes) and some of the Hsp150 was mature (Fig. 19 A, lane 3 and 4). Compared to the extremely fast Hsp150 transport without BFA (see Fig. 14 A), the addition of BFA blocked main part of the Hsp150 transport at the ER-exit. This result is in agreement with Yahara *et al.* (2001) studies with different Arf1p mutants in ARF2-deletion background. In *arf1-11* mutant all soluble medium proteins transport was blocked.

4.6.2.2 BFA in secretion mutants

We induced the BFA block also in the *sec7-4* mutant strain and studied Hsp150 transport, comparing to control reactions done without BFA addition under restrictive conditions (Fig. 19B). Without BFA, after pulse-labelling and immunoprecipitation Hsp150 was mainly in the mature cell-associated form but also a clear signal of an 89 kD ER form was seen. During chase the ER form maturated but no other change was seen. With the BFA treatment, two major changes were observed: Hsp150 maturation was blocked at the ER exit level and the Hsp150 protein was suddenly glycosylated like in the *sec23-1* mutant strain after one hour chase (see Fig. 13, lane 7). This massive glycosylation was observed already in the pulse sample and no other form, such as the 89 kD form was seen. Normally, extra glycosylation took place during the chase. BFA seemed to be able to load the Golgi transferases to the ER, and lead to Hsp150 glycosylation in a similar fashion as in the COPII component *sec23-1* mutant strain (see Fig. 13, lane 7). The same was observed in other secretion mutants like *ypt1-1* (Fig. 15) and *ypt6* (data not shown).

A similar treatment was performed in the COPI mutant *sec21-1* strain, under restrictive conditions (Fig. 19C). Without BFA Hsp150 matured and was partially transported to the culture medium as expected, since Hsp150 transport was COPI independent. The ER form was also detected and it developed its glycans as in the *sec18-1* strain (Fig. 13 lanes 5 and 6) due to blocked retrotransport. With the BFA treatment, we could block Hsp150 maturation and the protein remained in the ER form. Furthermore, Hsp150 glycans were not extended in the COPI-mutant like they were in the *sec7-4* mutant.

We concluded from BFA experiments that Hsp150 transport can be blocked by the addition of BFA to the ER exit step. Sec7p *in vivo* had no influence on Golgi to ER retrotransport, since the *sec7-4* mutant, where the mutation is in the active centre of ARF activation, did not prevent the merging of Golgi resident glycosyltransferase to the ER in an active form. In *in vitro* studies Spang and coworkers (2001) came to the same conclusion: Sec7p was not controlling retrotransport. Sec7p is not the retrotransport GEF although it blocked the ER exit of many proteins, like CPY, Gas1p and α factor (Franzusoff and Schekman

1989, Wolf *et al.* 1998). The ER exit block induced by the Sec7p mutation must take place in the anterograde transport to the other cargo proteins tested. The second transport block is induced by the mutant form of Sec7p at the level of mature protein transport after Kex2 cleavage, based on mature Hsp150 intracellular form (Fig. 13 lane 8; Fig. 19 B left panel).

Also, since Hsp150 transport is independent of COPI directed retrotransport, the BFA induced block to the Hsp150 ER exit blocked something else than COPI priming. It seemed that ARF activation is crucial for

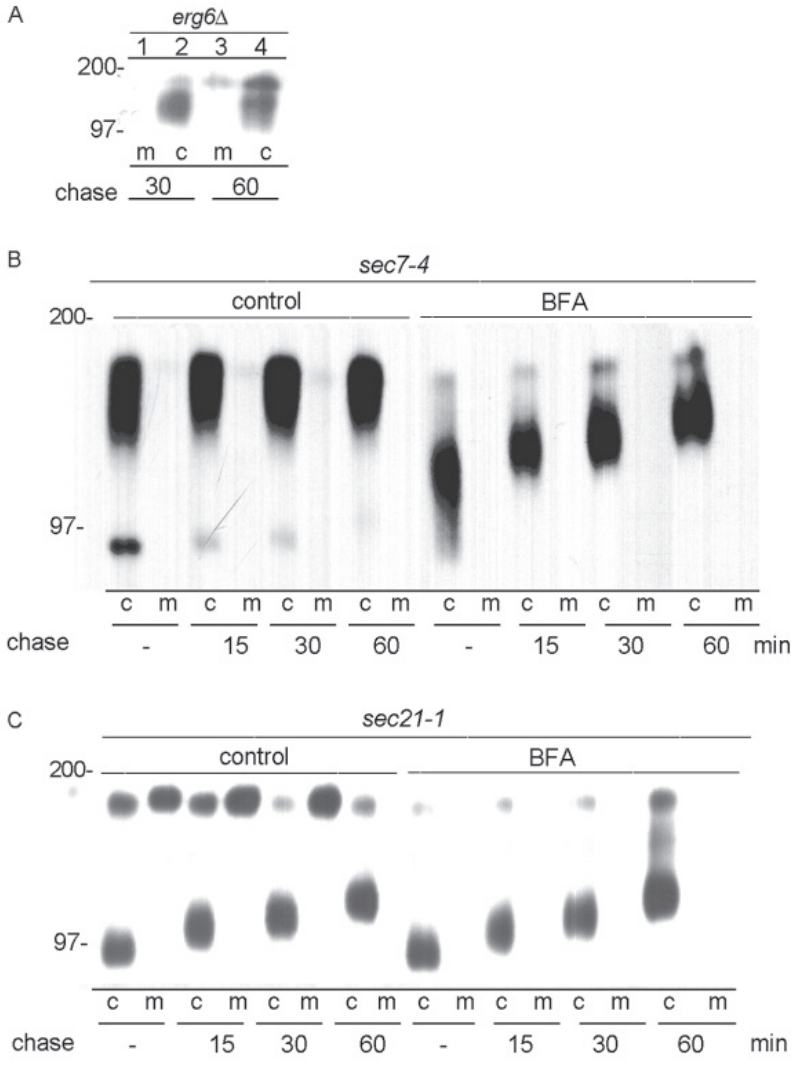

Figure 19. Effects of BFA to protein secretion in different cell lines. A) Hsp150 secretion from secretion wild type, ergosterol deficient yeast strain (H975). Cell samples (25x 10^6/500 µl) were incubated for 10 minutes at 37ºC and then 10 minutes before 5 minute metabolic labeling, 10 µl BFA (10 mg/ml) diluted to ethanol was added. Different chase times (30, 60 minutes) are indicated. Cell lysate (c, lanes 2 and 4) and medium (m, lanes 1 and 3) samples were immunoprecipitated with Hsp150 antiserum before SDS-PAGE. Migration of marker proteins (kDa) is indicated on the left. B) BFA treatment in the *sec7-4* strain (H11). After secretion block establishment by incubation for 10 minutes at 37 ºC either 10 µl ethanol (left panel) or BFA (10 mg/ml, right panel) was added and incubation contioned for 10 more minutes before metabolic labelling and CHX chase. After different chase times (0, 15, 30, 60 minutes as indicated) cell lysates (c) and culture media (m) were immunoprecipitated with Hsp150 antibody. C) Similar BFA treatment as in panel B was done in *sec21-1* cells.

the completion of ER exit either directly or indirectly. It may be that with BFA, we are able to block or at least severely retard the other, unknown COPI independent retrotransport route. This COPI independent route is also independent on Sec7p function or the function of Gea1p/Gea2p, since in both mutants Hsp150 is in the mature form after a ten minute pulse, but the transport of Hsp150 is not necessary independent of all of them. This putative retrotransport route to the ER has to originate from the Golgi/endosome remnant in COPI mutant conditions, as discussed before.

A second possibility for the BFA induced Hsp150 transport block is that ARF has other downstream effectors than COPI. ARF is known to activate phospholipase D (PLD1; Brown *et al*. 1993), which produces phosphatidic acid by hydrolyzing phosphatidylcholine. The PLD1 protein is only essential for yeast in the meiotic cell divisions at sporulation (Rudge *et al*. 1999, Xie *et al*. 1998). But this still remains a possibility, since Hsp150 is not essential either.

ARF activation might lead to COPI polymerization onto transport containers after COPII coat dissociation also in yeast cells as it does in mammalian cells. COPI polymerization may be important for the ER exit of a subset of proteins and the block induced by COPI mutants may take place also directly in the anterograde transport. The EM-results obtained by Rambourg and coworkers (2001) using the retrotransport mutant *sec21-3* also suggested that the transport block had to be after COPII function but before the completion of ER exit. Rambourg *et al*. saw ER-connected anastomosed ribbons connecting nuclear and peripheral ER, but no Golgi structures after a 20 minute incubation at restrictive temperature. After retuning to permissive temperatures ribbons changed gradually to secretion granules by the accumulation of glycosylated material into intersections of ribbon elements. It seems that material previously located in the Golgi is directed to the vacuole during the secretion block and newly synthesized material is selectively blocked at the ER exit. This ER exit block seems to take place after COPII function, since vesicle formations were not observed, when the block was released, as it was with other mutants, like *sec18-1* (Morin-Ganet *et al*. 2000). If this holds true, vesicle formation in yeast ER exit is not necessary.

Definitely, COPI functioned in glycosyltransferase recycling from the Golgi to the ER and seemed to be essential for it. Och1p immunofluorescence staining done by Gaynor and Emr (1997) in the *sec21-3* mutant supported our findings on the exclusive role of COPI in the *cis*-Golgi glycosyltransferase retrotransport. In mammalian cells, the immediate effect of BFA is COPI dissociation from the membranes to the cytosol (Lippincott-Schwartz *et al.* 1990). We saw that the functional coatomer is essential for the fusing of Golgi enzymatic contents to the ER, since merging of the *cis*-Golgi with the ER content did not take place after BFA treatment in non-functional *sec21-1* conditions. Maybe COPI is essential for SNARE harvesting or activation at the tip of the Golgi tubule, since fusion did not occur based on the glycosylation of the Hsp150 ER-form.

In BFA-treated mammalian cells Sec13p was still recruited to ER exit sites. COPII coats in ER exit sites were able to form a post exit site that did not mature to functional VTC without COPI function (Ward *et al.* 2001). Zhao and coworkers (2006) showed that GBF1 (a mammalian Gea1p and Gea2p homolog) was stably associated to Golgi membranes by BFA treatment also when membranes tubularize and merge to the ER. GBF1 associated also to peripheral puncta close to ER exit sites. By microinjecting anti-GBF1 antibody they dislocated COPI to the cytosol. GBF1 seemed to be the responsible GEF for ARF activation leading to COPI polymerization onto VTC precursors and for VTC maturation (Zhao *et al.* 2002).

In yeast Sec7p location is mainly with the *trans*-Golgi marker (Rossanese *et al.* 1999). Sec7p function was needed in *in vitro* produced vesicle fusion to the Golgi (Lupashin *et al* 1996). Also Sec7 domain peptide added to *in vitro* vesicle budding reaction inhibited cargo containing vesicle formation while a reverse sequence peptide

did not (Deitz *et al.* 2000). It seemed that the more stable location was in the *trans* Golgi, but still the function was needed also in the early transport pathway. Furthermore, in pull down assays Deitz and coworkers (2000) were able to show an interaction between the COPII coat component Sec23/24p and the C-terminal domain of the Sec7 protein.

4.6.3 Interaction of Sec7p and COPII coat component Sec24p

We wanted to see, if Sec7p and the coat component Sec24p really interacted directly in the cell. We used a two-hybrid approach. Sec24p was divided into three different pieces: N-terminus (amino acids 1-181), β-barrel-trunk-zinc-finger (amino acids 182-456) and α helical gelsolin domain (amino acids 457- 746) and fused to the transcription factor GAL4 DNA binding domain. The C-terminal part of the Sec7 protein we fused in three different pieces to the transcription factor GAL4 activation domain (amino acids 1709-1771, 1772-2009 and 1941-2009). Constructs were transformed into yeast cells in different combinations and the ability to activate the GAL4 promoter was tested by the activity of β-galactosidase whose expression was under the control of the promoter. None of the constructs on its own was able to induce the promoter (not shown). Only the Sec24p β-barrel-trunk-zinc-finger domain (Fig. 20 B) with the Sec7p domain 1772-2009 (Fig. 20 E) was able to interact to produce galactosidase activity. The interaction was weak, but specific. Comparing different Sec7 homologs in different species from

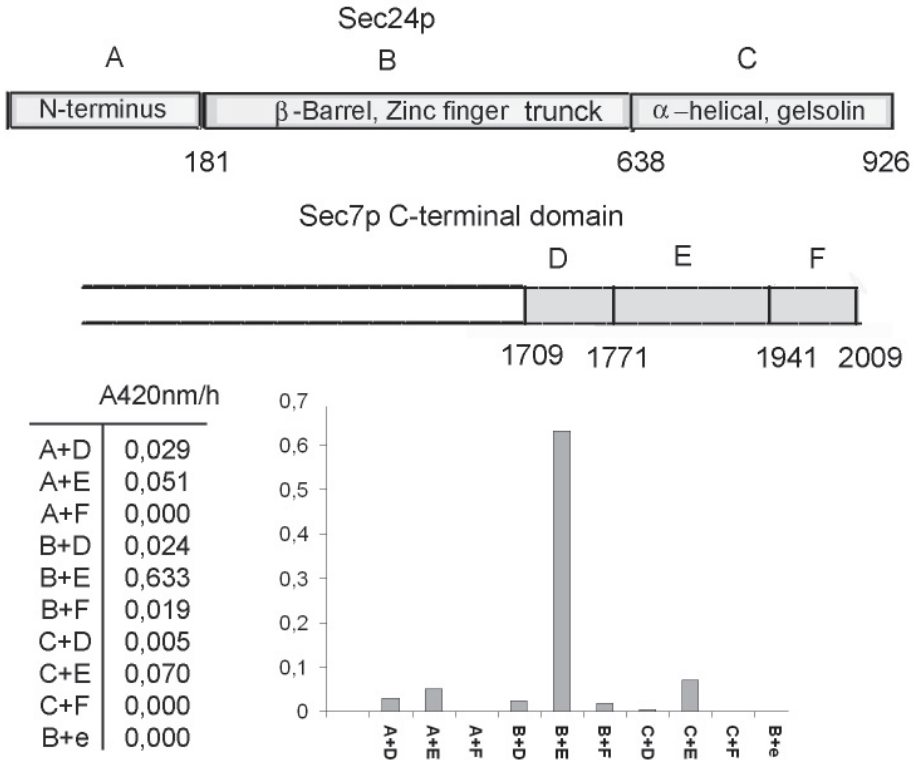

Figure 20. Two-hybrid interactions between Sec24p and Sec7p. Sec24p was fused with the GAL1 promoter upstream activator sequence binding domain of GAL4 as a bait-protein in three different pieces indicated as A) N-terminal 180 amino acids, B) β-barrel, trunk, zinc finger domains (aa 181-637 from Sec24p) and C) last 288 amino acids forming α-helical and gelsolin domains. C-terminal domain fragments of Sec7p were fused as prey constructs to GAL4 activation domain containing a nuclear localization signal from SV40 and a HA-tag aa 1709-1771 D), aa 1772- 2009 E) or aa1943-2009. LacZ activities induced by different fusion proteins combinations are indicated with capital letters. The activities were counted by ONPG substrate breakdown measured by absorbance at 420 nm from 1ml reactions containing 10^8 cells in one hour.

human to plants (35 species; Combet *et al*. 2000), almost all had a conserved PNLLKQE sequence at the C-terminus (in yeast Sec7p amino acids 1987-1993). When we changed the conserved leucines to less hydrophobic glycines (Fig. 20 e) and tested the interaction with Sec24p middle domain (Fig. 20 B), the interaction was lost. Our resut indicated that C-terminal domain of Sec7p directly interacted with COPII coat component Sec24p.

4.6.4 Sec7p interaction leads to COPI anterograde coat polymerization?

The interaction of Sec7p with the COPII component Sec24p might be important for continued protein transport. Weak interaction suggests its transient nature. The interaction between Sec7p and Sec24p must take place at the early phases of the transport. Clearly, the mutant form of Sec7p inhibited ER exit (except for Hsp150), and the inhibition seemed to take place before COPII coated vesicles shed their coat and, based on glycosylation defects, before the ER exit was completed. This interaction may be important later for Arf1p activation and lead to COPI coat polymerization which might be essential for the ER exit of a subset of proteins.

Two mutants of *SEC21* behaved also partially in a different way in Hsp150 and its variant transport that might indicate a role for the COPI coat in the anterograde transport. The retrotransport mutant *sec21-3* did not inhibit the transport of Hsp150ΔBla (III Fig 3A) but a portion of Hsp150 was in the ER form in the *sec21-1* mutant (Fig. 19 C) that was isolated as an anterograde transport mutant. This may be interpreted as a direct defect in the ER exit. If different populations of COPII coated vesicles are formed from ER, and one of those needed Sec7p contact leading to COPI polymerization to complete the ER exit. A portion of Hsp150 was captured also into these vesicles that were unable to mature in *sec21-1* mutant, while a second population maturated directly without the COPI function. Early Golgi markers dispersed in vesicles unable to fuse to ER without functional COPI coat and later Golgi contents fused to prevacuole/vacuole, from were another retrotransport cycled reusable proteins back to ER.

5 CONCLUSIONS

The nonessential yeast glycoprotein Hsp150 has during protein evolution developed at least two different features in one polypeptide that ensure its ER exit: the repetitive region and the C-terminal domain. The repetitive region directed Hsp150 to normally composed COPII vesicles at the ER exit in a selective and active fashion. The degree of selectivity suggests that the Hsp150 transport is receptor mediated. Hsp150 secretion continued with similar kinetics for two to three hours when subsets of proteins were retarded due to the non-functional COPI. The COPI mediated retrotransport was needed to recycle the active *cis* Golgi located glycosyltransferases through the ER. The continuous Hsp150 secretion under non-functional COPI condition also suggested the existence of another retrotransport route than COPI mediated, which replenished essential components (SNAREs) for the Hsp150 ER exit.

BFA treatment, which locked ARFs in a GDP-bound inactive form to membranes, suggested ARF-mediated priming also for the other putative COPI independent retrotransport route that most probably originated beyond *trans* Golgi at least in COPI mutant conditions. A second possibility is that ARF is directly needed for ER-exit and that a subset of proteins also required the functional COPI coat after COPII dissociation for complete ER-exit. ARF's GTP exchange factor, essential Sec7p, seemed to have a dual role inside the cell: in late Golgi exit and before anterograde transport vesicle fusion to Golgi. Our results suggested a transient interaction between the Sec7p and the ER vesicle forming coat COPII, placing the need for Sec7p very early, before the COPII vesicles shed their coat.

Furthermore, the extremely fast transport rate of Hsp150 out of the ER through the Golgi compartment to the mature form suggests the existence of a fast track transport route in yeast cells that achieves the Golgi maturation through another route than the cisternal maturation route. In the cisternal maturation, all proteins captured to the forming *cis* Golgi cisterna acquire the Golgi modifications in the same container, which slowly adjusts its enzyme contents by retrotransport from later Golgi compartments. Our estimation of Hsp150 transport rate from the ER through the Golgi is approximately ten-fold faster than estimations of the cisternal maturation rate.

The fastest ER exit and transport rate is achieved by the endogenous Hsp150, as compared with its fusion with other proteins; a major portion (76%) of Hsp150 is externalized from the ER in less than 60 seconds. For this extremely fast transport rate the protein needed its C-terminal domain. We found an ATPase activity in the C-terminal domain, which was responsible for the ER-exit in yeast strains where normal COPII coat function was compromised (thermosensitive mutants of Sec13p and Sec24p). When the ATPase activity was abolished by a point mutation in the conserved Walker A motif, the protein remained in the ER under non-functional COPII conditions.

The Sec13p independent transport was selective and active in the sense that proteins fused to the C-terminal domain of Hsp150 without the repetitive region were directed out of the ER under restrictive conditions. Thus, retention was not the reason why other proteins were not transported under non-functional COPII conditions. Also, Sec13p and Sec24p independent transport must be receptor mediated due to the selectivity dictated by the ATPase activity. As Hsp150 is a lumenal soluble protein and the coat polymerization to the membrane is cytosolic, a transmembrane connection is certainly needed.

The known cuboctahedron structure of the outer layer of the COPII coat in which Sec13p has an essential connecting role suggested that Sec13p can not be missing from the coat formation. Still, a deletion strain of Sec13p exists where the null mutation of Sec13p is compensated by additional bypass sec thirteen mutations (Bst). We propose that the ATPase activity directed the Hsp150 ER exit to transport containers where both Sec24p and Sec13p might be replaced by homologs, avoiding the barriers set by Bst proteins against

anomalous coat formation. This COPII-like coat structure might be formed at least under heat shock conditions. It is not known whether other proteins than Hsp150 and SNAREs are also included in these vesicles. If those coat structures are formed exclusively for Hsp150 transport, the role of Hsp150 in nature and during evolution has been far more important than it is in the laboratory strains of baker's yeast. By studying the transport of a nonessential glycoprotein out of the simple eukaryotic model organism *Saccharomyces cerevisiae* we found evidence of the variation of the basic transport machinery both in the anterograde and in the retrograde transport between the ER and the Golgi. It remains to be seen, whether these alternate transport routes also exist in higher eukaryotic cells.

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